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(54) Title: USE OF DNA ENCODING OSTEOPROTEGERIN TO PREVENT OR INHIBIT METABOLIC BONE DISORDERS

(57) Abstract: The invention provides a method to inhibit or prevent osteoclastogenesis by expressing osteoprotegerin in mammalian cells.

USE OF DNA ENCODING OSTEOPROTEGERIN TO PREVENT OR INHIBIT METABOLIC BONE DISORDERS

Background of the Invention

Living bone tissue exhibits a dynamic equilibrium between deposition and resorption of bone. These processes are mediated primarily by two cell types: osteoblasts, which secrete molecules that comprise the organic matrix of bone; and osteoclasts, which promote dissolution of the bone matrix and solubilization of bone minerals. In young individuals with growing bone, the rate of bone deposition exceeds the rate of bone resorption, while in older individuals and in several bone disorders the rate of resorption can exceed deposition. In the latter situation, the increased breakdown of bone leads to reduced bone mass and strength, increased risk of fractures, and slow or incomplete repair of broken bones.

Osteoclasts are large phagocytic multinucleated cells which are formed from hematopoietic precursor cells in the bone marrow. Although the growth and formation of mature functional osteoclasts is not well understood, it is thought that osteoclasts mature along the monocyte/macrophage cell lineage in response to exposure to various growth-promoting factors. Early development of bone marrow precursor cells to preosteoclasts are likely to be mediated by soluble factors such as tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-6 (IL-6), and leukemia inhibitory factor (LIF). In culture, pre-osteoclasts are formed in the presence of added macrophage colony stimulating factor (M-CSF). These factors act primarily in early steps of osteoclast development. The involvement of polypeptide factors in terminal stages of osteoclast formation has not been extensively reported. It has been noted, however, that parathyroid hormone stimulates the formation and activity of osteoclasts and that calcitonin has the opposite effect, although to a lesser extent.

Several metabolic bone disorders share a common final pathogenic pathway consisting of a relative or absolute osteoclastic overactivity. This

enhanced activity results in excessive bone matrix resorption and loss, ultimately leading to the clinical complications of skeletal fragility, deformity, pain, and fracture (Einhorn et al., 1996). The plasma cell malignancy multiple myeloma is a striking example of this process; 80% of patients present with bone pain (Mundy et al., 1989), and bone destruction accounts for the most debilitating features of this disease, including intractable pain, pathologic fractures, and the constellation of symptoms associated with hypercalcemia (Roodman et al., 1997). A number of cytokines, including IL-1, IL-6, TNF-α, lymphotoxin, and M-CSF (Bataille et al., 1992; Gozzolino et al., 1989), have been implicated in the pathogenesis of myelomatous bony lesions through differentiation of osteoclast precursors and activation of mature osteoclasts (Figure 1) (Roodman, 1997). This enhanced osteoclastic activity gives rise to diffuse, severe osteopenia and/or discrete osteolytic lesions throughout the skeleton, thereby threatening its structural integrity (Foerster et al., 1999).

Treatment of bony lesions in multiple myeloma has so far been limited to systemic chemotherapy and/or local external beam radiation therapy (Foerster et al., 1999), however, these treatments induce severe side effects, e.g., myelosuppression and are accompanied by nausea, vomiting, weight loss, malaise and fatigue. These treatments also often require hospital admission and incur a considerable cost. Moreover, even with chemotherapy-induced remissions, bone healing is observed in only 30% of patients (Rodriguez et al., 1972). The chemotherapy regimens for multiple myeloma also often contain very high doses of glucocorticoids (Foerster et al., 1999), which can cause additional bone loss. Bisphosphonates, such as oral clodronate and intravenous pamidronate, have been used for myelomatous bone lesions. Although these agent have been found to be generally effective, pamidronate can cause flu-like symptoms during infusion, and a single 90 mg treatment is quite costly (The Medical Letter, 1998). The therapeutic options for osteolytic metastatic lesions from other malignancies, such as breast cancer, are even more limited and, thus far, consist only of regional radiation therapy and systemic chemotherapy.

Other bone disorders that currently have limited treatment options include Paget's disease and osteoporosis. Severe Paget's is an incapacitating condition which can only be partially treated with repeated doses of

bisphosphonates. It is estimated that 1.5 million fractures attributable to osteoporosis occur annually in the United States, incurring a total cost estimated at 13.8 billion dollars in 1995 alone (Ray et al., 1997). Although several new medications have become available during the last several years for osteoporosis, and some can produce a transient rise in bone mineral density, all of them are antiresorptive agents that only slow the rate of loss in the long-term, and none can replace most of the bone that has already been lost.

Thus, what is needed is a method to inhibit or prevent bone diseases associated with decreased bone mass or density.

Summary of the Invention

The present invention provides a method to inhibit, prevent or reverse a metabolic bone disorder or condition associated with aberrant or altered osteoclastogenesis, such as a disorder or condition characterized as having a net bone loss, e.g., osteopenia and localized osteolysis. For example, multiple myeloma is a condition associated with increased osteoclastic activity resulting in osteolytic lesions and hypercalcemia. The method comprises contacting mammalian cells with an amount of a composition comprising a nucleic acid molecule encoding osteoprotegerin, a variant thereof, or a biologically active fragment thereof, so as to yield genetically altered (e.g., transduced or transfected) cells comprising the nucleic acid molecule. The genetically altered cells can be introduced into a mammal so as to alter osteoclastogenesis in that mammal. Thus, the introduction of cells that express osteoprotegerin, e.g., in the bone microenvironment, can increase local levels of osteoprotegerin and thus inhibit, prevent or reverse osteoclastic overactivity, e.g., prevent, inhibit or reverse the bony lesions induced by myeloma cells or solid tumor osteolytic metastases.

The mammalian cells to be altered are preferably pluripotent cells such as bone marrow cells which comprise mesenchymal progenitor cells, e.g., isolated unfractionated bone marrow, or isolated mesenchymal precursor (progenitor) cells, although other cell types may be useful in the methods of the invention, including monocyte/macrophage precursors and other marrow cells, fibroblasts, keratinocytes and various neo-organoids, as well as tumor cells or tumor cell lines, e.g., human melanoma cells, human myeloma cells or breast cancer cells.

Mesenchymal progenitor cells can be isolated from other marrow cells, and can be efficiently expanded *in vitro* (Hou et al., 1999). These cells can also be genetically altered, and when transplanted retain the ability to differentiate into mature bone cells (Allay et al., 1997). Moreover, intravenous infusion of human mesenchymal stromal cells resulted in engraftment of these cells to the bone marrow space (Horwitz et al, 1999).

Thus, a preferred method of the invention comprises contacting *ex vivo* a population of cells comprising mesenchymal progenitor cells, e.g., cells harvested from at least one mammal, with a composition comprising a nucleic acid molecule encoding osteoprotegerin, a variant thereof, or a biologically active fragment thereof, so as to yield a population comprising cells that are genetically altered with osteoprotegerin nucleic acid. The cells may be introduced to a mammal, preferably after expansion of the genetically altered cells *in vitro*. Thus, the invention provides for genetically altered allogeneic or autologous mammalian cells which may be introduced to a mammal, for example, by intravenous infusion. The use of autologous cells eliminates the need for the chronic immunosuppression required by allogeneic transplants. Additionally, when responding to osteoclastic bone resorption, genetically altered mesenchymal cells locally differentiate into osteoprotegerin expressing osteoblasts, thus increasing osteoprotegerin production in the relevant bone microenvironment.

The composition of the invention preferably comprises a nucleic acid molecule which comprises a transcriptional regulatory region, element or sequence, e.g., a promoter and optionally an enhancer, operably linked to a nucleic acid sequence which encodes osteoprotegerin. Preferred compositions which are contacted with cells include recombinant virus, e.g., replication incompetent recombinant retrovirus, recombinant lentivirus, recombinant adenovirus, and recombinant adeno-associated virus, which comprise the nucleic acid molecule encoding osteoprotegerin, although cells may be contacted with a composition comprising isolated and purified nucleic acid encoding osteoprotegerin, e.g., a plasmid.

The invention also provides a therapeutic method in which a composition of the invention is administered to a mammal in need thereof in an amount

effective to inhibit, prevent or treat a condition associated with altered osteoclastogenesis.

The expression of osteoprotegerin in a mammal after transplant of cells subjected to *ex vivo* manipulation or after *in vivo* introduction of a composition of the invention, allows for a significantly reduced dosing frequency, with potential for several weeks between each administration, and even single-dose treatment, due to stable osteoprotegerin serum levels, thereby avoiding the frequent peaks and troughs consequent to the short circulating half-life of osteoprotegerin, and in contrast to parenteral administration of the protein (Simonet et al., 1997).

Further, targeting of osteoprotegerin expression to the bone marrow microenvironment through tissue-specific transcriptional promoters or *ex vivo* transduction of cells with known tissue tropism (e.g., mesenchymal or hematopoietic progenitor cells with tropism for the medullary compartment) may be useful. Cell-, tissue-, or organ-specific expression of osteoprotegerin may be accomplished by employing a cell-, tissue-, or organ-specific transcriptional regulatory element, sequence or region, such as with an ApoE promoter for hepatic expression (Simonet et al., 1997) or an osteocalcin promoter for skeletal expression. The use of a skeletal-specific promoter may result in elevated osteoprotegerin levels in the bone microenvironment, while avoiding undue elevations in systemic osteoprotegerin concentrations, which could lead to osteopetrosis. Other preferred transcriptional regulatory elements are those comprising chemically responsive regulatory sequences, for example, regulatory sequences responsive to tetracycline/doxycycline, to dexamethasone (e.g., the MMTV LTR), and rapamycin or analogs thereof (Ariad, Boston, MA).

Therefore, the present invention provides a therapeutic method to prevent, inhibit or treat bone diseases or conditions caused by osteoclastic overactivity, e.g., osteolytic metastatic lesions and hypercalcemia in multiple myeloma patients, and other hematologic malignancies and solid malignancies, involutional osteoporosis, Paget's disease of bone, osteopenia associated with inflammatory arthropathies, glucocorticoids, and other immunosuppressants, bone loss related to prolonged skeletal unloading, bone resorption due to

refractory hyperparathyroidism, such as with chronic renal insufficiency and unresectable parathyroid carcinoma and the like.

Brief Description of the Figures

Figure 1 shows the cytokine network in myeloma bone disease. Cytokines produced by myeloma cells, marrow stromal cells, osteoblasts, and osteoclasts enhance the growth of myeloma cells and osteoclast formation.

Figure 2 depicts mechanisms of osteoprotegerin (OPG) and the ligand thereof OPGL in regulating osteoclastogenesis. (1) Stromal cells and mature osteoblasts express cell membrane-bound OPGL (cOPGL) and soluble OPGL (sOPGL). (2) Stromal cells and mature osteoblasts secrete OPG, a soluble OPGL antagonist. (3) Both forms of OPGL bind to their cognate receptor, osteoclast differentiation activator receptor (ODAR), located on osteoclast precursor cells and in the presence of permissive factors such as CSF-1, promote osteoclastogenesis. (4) OPG counteracts the effects described in (3) by competing for and neutralizing OPGL (Hofbauer et al., 1998).

Figure 3 depicts exemplary vectors for use in the methods of the invention. 1) A plasmid vector. 2) A lentivirus vector. 3) An adenovirus shuttle vector (Quantum Biotechnologies, Montreal Canada).

Figure 4 shows the correlation between OPG expression and viral dose in infected ARH-77 cells.

Figure 5 shows OPG expression over time in infected ARH-77 cells.

Figure 6 depicts survival times in treatment versus control groups.

Figure 7 shows total body bone mineral density (BMD) in control mice (left-hand column), negative control mice (uninjected, middle column), and treatment mice (right-hand column).

Figure 8 shows femur BMD in control mice (left-hand column), negative control mice (uninjected, middle column), and treatment mice (right-hand column).

Figure 9 shows tibia BMD in control mice (left-hand column), negative control mice (uninjected, middle column), and treatment mice (right-hand column).

Figure 10 shows vertebral BMD in control mice (left-hand column), negative control mice (uninjected, middle column), and treatment mice (right-hand column).

Detailed Description of the Invention

Definitions

As used herein, "a condition associated with aberrant or altered osteoclastogenesis" includes, but is not limited to osteoclastic bony lesions, e.g., lesions associated with solid tumors such as breast cancer, Paget's disease, involutional osteoporosis, myeloma osteolysis and hypercalcemia, osteopenia associated with arthropathies, glucocorticoids and other immunosuppressants, bone loss related to prolonged skeletal unloading, and bone resorption due to refractory hyperparathyroidism, e.g., in chronic renal insufficiency and unresectable parathyroid carcinoma, as well as any other condition characterized by either relative or absolute osteoclast overactivity.

As used herein, "pluripotent" cells are cells which have multiple developmental of functional capacities, i.e., the cells are capable of developing into more than one cell type, tissue or organ. Pluripotent cells are not totipotent cells, i.e., capable of developing into any cell of an organism. A fertilized ovum is a totipotent cell. Preparations of mesenchymal precursor cells and bone marrow cells contain pluripotent cells.

As used herein, the terms "isolated and/or purified" refer to *in vitro* isolation of a nucleic acid, e.g., DNA molecule from its natural cellular environment, and from association with other components of a cell or virus, such as nucleic acid or polypeptide, so that it can be sequenced, replicated, and/or expressed. For example, "isolated nucleic acid" is RNA or DNA containing greater than about 50, preferably about 300, and more preferably about 500 or more, sequential nucleotide bases that encode osteoprotegerin, or a variant thereof, or a biologically active portion thereof, that is complementary or hybridizes, respectively, to osteoprotegerin nucleic acid and remains stably bound under stringent conditions, as defined by methods well known in the art, e.g., in Sambrook et al. (1989). The "biological activity" of nucleic acid encoding osteoprotegerin, a variant or a portion thereof can be measured by methods known to the art including, but not limited to, methods described

herein. Thus, the RNA or DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA or DNA and is preferably substantially free of any other RNA or DNA. The phrase "free from at least one contaminating source nucleic acid with which it is normally associated" includes the case where the nucleic acid is reintroduced into the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell, e.g., in a viral vector or plasmid. An example of isolated nucleic acid within the scope of the invention is nucleic acid that encodes a polypeptide having at least about 80%, preferably at least about 90%, and more preferably at least about 95%, amino acid sequence identity with human osteoprotegerin.

As used herein, the term "recombinant nucleic acid" e.g., "recombinant DNA sequence or segment" refers to a nucleic acid, e.g., to DNA, that has been derived or isolated from any appropriate viral or cellular source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome which has not been genetically altered with exogenous DNA or in a non-recombinant viral genome. An example of DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment within a given virus or organism, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from the source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering. Thus, recovery or isolation of a given fragment of DNA from a restriction digest can employ separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. See Lawn et al. (1981) and Goeddel et al. (1980). Therefore, "DNA" includes completely synthetic DNA sequences, semi-synthetic DNA sequences, DNA

sequences isolated from biological sources, and DNA sequences derived from RNA, as well as mixtures thereof.

A "variant" nucleic acid molecule is a molecule having at least one base substitution (relative to another molecule, generally a "wild-type" nucleic acid sequence). Variant nucleic acid molecules may be are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the nucleic acid molecule.

Osteoprotegerin

DNA encoding osteoprotegerin has been described (see, for example, Simonet et al. (1997) and published international application No. WO 96/26271). Osteoprotegerin dramatically increases the bone density in transgenic mice overexpressing osteoprotegerin, which mice displayed a non-lethal osteopetrosis that was characterized by increased bone density and decreased osteoclast differentiation (Simonet et al., 1997). Moreover, exogenous administration of osteoprotegerin has been shown to increase bone density in normal mice, as well as to protect rats against ovariectomy-induced bone loss (Simonet et al., 1997). Nevertheless, exogenous osteoprotegerin therapy shows some limitations, such as the short half-life of the endogenous osteoprotegerin protein and frequent parenteral administration.

In contradistinction, osteoprotegerin knockout mice develop increased bone resorption and severe osteoporosis (Mizuno et al., 1998; Bucay et al., 1998). An analysis of osteoprotegerin activity in *in vitro* osteoclast formation revealed that osteoprotegerin does not interfere with the growth and differentiation of monocyte/macrophage precursors, but likely blocks the differentiation of osteoclasts from monocyte/macrophage precursors. Thus, osteoprotegerin appears to have specificity in regulating the extent of osteoclast formation.

Osteoprotegerin, whose human gene is on chromosome 8q23-24, is a disulfide-linked homodimer of 110 kDa with 4 tandem cysteine-rich TNF receptor motifs at the N-terminus, as well as 2 death domain homologous

regions. The amino-terminal domain spanning about residues 22-194 of the full-length polypeptide (the N-terminal methionine is designated residue 1) shows homology to other members of the tumor necrosis factor receptor (TNFR) family, especially TNFR-2, through conservation of cysteine rich domains characteristic of TNFR family members. The carboxyl terminal domain spanning residues 194-401 has no significant homology to any known sequences. Unlike a number of other TNFR family members, osteoprotegerin has no lipophilic domain and appears to be exclusively a secreted protein and does not appear to be synthesized as a membrane associated form.

The ligand for osteoprotegerin is called OPGL (Lacey et al., 1998) (also referred to as TNF-related activation-induced cytokine (TRANCE) and receptor activator of NF-κB ligand (RANKL)). OPGL, which belongs to the type II class of transmembrane proteins, exists in a cell-bound and soluble C-terminal form and is highly expressed in osteoblast and bone stromal cells (Kong et al., 1999). This expression is regulated by known bone-resorting factors such as vitamin D, IL-11, prostaglandin E_2 (PGE_2), and parathyroid hormone (PTH) (Yasuda et al., 1998). OPGL binds to the cell surface of osteoclastic lineage cells and, in the presence of the permissive factor colony-stimulating factor-1 (CSF-1), induces osteoclastogenesis *in vitro* in the absence of stromal cells, glucocorticoids and vitamin D, factors previously considered essential for osteoclastogenesis (Lacey et al., 1998; Yasuda et al., 1998). OPGL stimulates bone resorption both *in vitro* and *in vivo* (Lacey et al., 1998). The latter, after subcutaneous injection of recombinant OPGL into normal mice, was associated with massive bone loss and profound rapid-onset hypercalcemia (Lacey et al., 1998).

Thus, OPGL and osteoprotegerin, through coupled but diametrically opposite functions, regulate bone mass by modulating osteoclastogenesis. OPGL appears to be the final common pathway through which multiple mediators produce bone resorption by osteoclast recruitment and activation, whereas osteoprotegerin functions as the naturally occurring soluble receptor that acts as a decoy for OPGL, thus counterbalancing its effects and preserving bone mass (Figure 2) (Hofbauer et al., 1998).

Nucleic Acid Molecules of the Invention

The invention provides for an isolated nucleic acid molecule encoding a polypeptide having at least one of the biological activities of osteoprotegerin. As described herein, the biological activities of osteoprotegerin include, but are not limited to, any activity involving bone metabolism and in particular, include increasing bone density or mass. Thus, the present invention employs a nucleic acid molecule comprising a nucleic acid sequence encoding osteoprotegerin, a variant thereof, or a biologically active fragment thereof. A nucleic acid molecule of the invention can be obtained from any vertebrate source, preferably a mammalian source. A preferred nucleic acid molecule of the invention includes a nucleic molecule encoding a mammalian osteoprotegerin, e.g., a human, non-human primate, rat, mouse or other mammalian osteoprotegerin, the complementary strands thereof, or a portion thereof, preferably including nucleic acids which hybridize under stringent conditions to human, rat or mouse osteoprotegerin DNA but which do not undergo detectable hybridization with other known members of the TNF receptor superfamily. Therefore, the invention provides for nucleic acids which encode rat, mouse and human osteoprotegerin as well as nucleic acid sequences hybridizing thereto which encode a polypeptide having at least one of the biological activities of osteoprotegerin.

Hybridization conditions for nucleic acids are described in Sambrook et al. (1989). The conditions for hybridization are generally of high stringency using temperatures, solvents and salt concentrations wherein the hybridizing sequences are about 12-20°C below the melting temperature (T_m) of the perfectly matched duplex. For example, high stringency hybridization conditions include 5xSSC, 50% formamide and 42°C (see Sambrook et al., 1989). Equivalent stringency to these conditions may be readily obtained by adjusting salt and organic solvent concentrations and temperature.

The length of hybridizing nucleic acids of the invention may be variable since hybridization may occur in part or all of the polypeptide-encoding regions, and may also occur in adjacent noncoding regions, i.e., hybridizing nucleic acids may be truncations or extensions of the sequences. Truncated or extended nucleic acids are encompassed by the invention provided they retain one or more

of the biological properties of osteoprotegerin. The hybridizing nucleic acids may also include adjacent noncoding regions which are 5' and/or 3' to the osteoprotegerin coding region. The noncoding regions may include regulatory regions involved in osteoprotegerin expression, such as promoters, enhancers, translational initiation sites, transcription termination sites and the like.

Examples of the nucleic acids of the invention include cDNA, genomic DNA, synthetic DNA and RNA. cDNA is obtained from libraries prepared from mRNA isolated from various tissues expressing osteoprotegerin. In humans, tissue sources for osteoprotegerin include kidney, liver, placenta and heart. Genomic DNA encoding osteoprotegerin is obtained from genomic libraries which are commercially available from a variety of species. Synthetic DNA is obtained by chemical synthesis of overlapping oligonucleotide fragments followed by assembly of the fragments to reconstitute part of all of the coding region and flanking sequences (see U.S. Patent No. 4,695,623 describing the chemical synthesis of interferon genes). RNA is obtained most easily by procaryotic expression vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

Variants of the Nucleic Acid Molecules of the Invention

Also provided by the invention are variants of osteoprotegerin nucleic acid sequences. As used herein, variants include nucleic acid sequences having a least one addition, substitution, insertion or deletion of nucleotides such that the resulting sequences encode polypeptides having one or more amino acid residues which have been added, deleted, inserted or substituted and the resulting variant polypeptide has the activity of osteoprotegerin. The nucleic acid variants may be naturally occurring, such as by splice variation or polymorphism, or may be constructed using site-directed mutagenesis techniques available to the skilled worker. It is anticipated that nucleic acid variants will encode amino acid changes in regions of the molecule which are least likely to disrupt biological activity. Other variants include a nucleic acid encoding a membrane-bound form of osteoprotegerin having an extracellular domain along with transmembrane and cytoplasmic domains. In one embodiment, variants include nucleic acids encoding truncated forms of osteoprotegerin having one or more amino acids deleted from the carboxyl terminus, e.g., nucleic acids encoding osteoprotegerin

having from 1 to 216 amino acids deleted from the carboxyl terminus. Also included are nucleic acids encoding truncated forms of osteoprotegerin having one or more amino acids deleted from the amino terminus, e.g., truncated forms including those lacking part of all the 21 amino acids comprising the leader sequence. Additionally, the invention provides for nucleic acids encoding osteoprotegerin having from 1 to 10 amino acids deleted from the mature amino terminus (at residue 22) and, optionally, having from 1 to 216 amino acids deleted from the carboxyl terminus (at residue 401). Optionally, the nucleic acids may encode a methionine residue at the amino terminus.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing variants. This technique is well known in the art as described by Adelman et al. (1983). Briefly, osteoprotegerin DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of osteoprotegerin. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the osteoprotegerin DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (1978).

The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera et al. (1987). Thus, the DNA that is to be mutated may be inserted into one of these vectors to generate single-stranded template. Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook et al. (1989).

Alternatively, single-stranded DNA template may be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of osteoprotegerin, and the other strand (the original template) encodes the native, unaltered sequence of osteoprotegerin. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutations(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thiodeoxyribocytosine called dCTP-(αS) (which can be obtained from the Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(αS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that

is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101.

The nucleic acid sequences of the invention may be used for the detection of osteoprotegerin sequences in biological samples in order to determine which cells and tissues are expressing osteoprotegerin mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to osteoprotegerin. Such screening is well within the capabilities of one skilled in the art using appropriate hybridization conditions to detect homologous sequences. The nucleic acids are also useful for modulating the expression of osteoprotegerin levels by anti-sense therapy or gene therapy. The nucleic acids are also used for the development of transgenic animals which may be used for the production of the polypeptide and for the study of biological activity.

Expression Cassettes, Vectors and Host Cells

An expression cassette and vector containing a nucleic acid sequence encoding osteoprotegerin, host cells transformed with the cassette or vector, and methods for the introduction of the cassette or vector into host cells are also provided by the invention. The expression cassette preferably comprises a recombinant DNA comprising a transcriptional regulatory region, e.g., a promoter, operably linked to a nucleic acid sequence encoding osteoprotegerin. Generally, the recombinant DNA sequence or segment is in the form of chimeric DNA. The vector may be prepared by introducing, i.e., linking, the cassette to another nucleic acid molecule, e.g., a plasmid or viral vector. A variety of plasmid vectors are available for expressing osteoprotegerin in host cells (see, for example, volume 185 in: Methods in Enzymology (1990)). Viral vectors, e.g., retroviral and adenovirus-based gene transfer vectors, may be used for the expression of osteoprotegerin in human cells, e.g., for gene therapy.

To prepare expression cassettes for introduction into cells, the recombinant DNA sequence or segment may be circular or linear, double-stranded or single-stranded. Preferably, the expression cassette of the invention comprises a transcription regulatory element, sequence or region, e.g., a promoter, transcription termination sequence, and/or enhancer, operably linked

the nucleic acid molecule encoding osteoprotegerin. For example, the expression cassette may comprise a promoter that is active in mammalian cells, or may utilize a promoter already present in the genome that is the transformation target. Such promoters include the CMV promoter, as well as the SV40 late promoter and retroviral LTRs (long terminal repeat elements), although many other promoter elements, such as those providing cell- or tissue-specific expression, e.g., the Apo(E) or osteocalcin promoter, well known to the art may be employed in the practice of the invention.

As used herein, "chimeric" means that a cassette or vector comprises DNA from at least two different species, or comprises DNA from the same species, which is linked or associated in a manner which does not occur in the "native" or wild type of the species.

Other elements functional in the host cells, such as introns and the like, may also be a part of the expression cassette. Such elements may or may not be necessary for the function of the expression cassette, but may provide improved expression cassette by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the expression cassette as desired to obtain the optimal performance of the recombinant DNA in the cell.

"Control sequences" is defined to mean DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotic cells, for example, include a promoter, and optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers. Control sequences also include sequences for viral replication. Thus, sequences directing expression and secretion of osteoprotegerin may be homologous, i.e., the sequences are identical or similar to those sequences in the genome involved in osteoprotegerin expression and secretion, or they may be heterologous.

"Operably linked" is defined to mean that the nucleic acid molecule is placed in a functional relationship with another nucleic acid molecule. For example, DNA for a presequence or secretory leader is operably linked to DNA for a peptide or polypeptide if it is expressed as a preprotein that participates in the secretion of the peptide or polypeptide; a promoter or enhancer is operably

linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

Vectors, e.g., plasmid or viral, for the expression of osteoprotegerin in cells contain at a minimum sequences required for vector propagation, e.g., a replication origin. These vectors also preferably include a selection marker or reporter gene, a promoter and a transcription termination site therefor. A selectable marker gene or a reporter gene facilitate identification and selection of genetically altered cells from the population of cells sought to be altered. Alternatively, the selectable marker or reported may be carried on a separate piece of DNA and used in a co-transformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide-resistance genes, such as *neo, hpt, dhfr, bar, aroA, dapA* and the like. See also, the genes listed on Table 1 of Lundquist et al. (U.S. Patent No. 5,848,956).

Reporter genes are used for identifying potentially transformed cells and for evaluating the functionality of regulatory sequences. Reporter genes which encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene which is not present in or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Preferred genes include the chloramphenicol acetyl transferase gene (*cat*) from Tn9 of *E. coli*, the beta-glucuronidase gene (*gus*) of the *uidA* locus of *E. coli*, and the luciferase gene from firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

Vectors suitable for expression in host cells are readily available and the nucleic acid molecule of the invention is inserted into the vectors using standard

recombinant DNA techniques. Vectors for tissue-specific expression of osteoprotegerin are also included. Such vectors include promoters which function specifically in liver, kidney, bone or other organs, and viral vectors, for the expression of osteoprotegerin in targeted in mammalian, e.g., human, cells.

Preferred vectors for use in the invention include viral vectors. For human gene therapy, it is desirable to use an efficient means of precisely inserting a single copy gene into the host genome. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into human cells. Other viral vectors are derived from poxviruses, Herpes simplex virus I, adenoviruses and adeno-associated viruses. Most of the current and proposed gene therapy clinical protocols employ retroviral vectors, although unlike lentivirus and adenovirus, retrovirus generally do not infect non-dividing cells.

Retroviruses are single-stranded RNA viruses which replicate viral RNA into DNA by reverse transcription. Upon replication in the host cell, the viral DNA is inserted into the host chromosome, where it becomes a provirus. Due to their efficiency at integrating into host cells, retroviruses are considered to be one of the most promising vectors for human gene therapy. These vectors have a number of properties that lead them to be considered as one of the most promising techniques for genetic therapy of disease. These include: (1) efficient entry of genetic material present in the vector into cells; (2) an efficient process of entry into target cell nucleus; (3) relatively high levels of gene expression; (4) minimal pathological effects on target cells; and (5) the potential to target to particular cellular subtypes through control of the vector-target cell binding and tissue specific control of gene expression.

Retroviral genomes consist of *cis*-acting and *trans*-acting gene sequences. The *cis* regions include the long terminal repeat (LTR) transcriptional promoter and DNA integration sites, the two primer binding sites required for reverse transcription of DNA from viral RNA, and the packaging signals required for efficient packaging of viral RNA into virions. The LTR is found at both ends of the proviral genome. *Trans*-functions include the proteins encoded by the *gag, pol*, and *env* genes, which are located between the LTRs. *Gag* and *pol* encode, respectively, internal viral structural and enzymatic proteins. *Env*

encodes the viral glycoprotein which confers infectivity and host range specificity on the virus. A retroviral vector generally consists of *cis* sequences and the replacement of the *trans* sequences with a gene(s) of interest. The *trans* functions can be provided by expression of the *trans* sequences in a helper cell or by a helper virus.

The general methods for constructing recombinant DNA which can genetically alter host cells are well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the DNA useful herein. For example, Sambrook et al. (1989) provides suitable methods of construction.

The vector can be readily introduced into the host cells, e.g., mammalian, bacterial, plant, yeast or insect cells, by any procedure useful for the introduction into a particular cell, e.g., physical or biological methods, to yield a genetically altered cell having the vector stably integrated into its genome or present as an episome which can persist in the genetically altered cells, so that the nucleic acid molecule of the present invention is maintained and/or expressed by the host cell.

Physical methods to introduce a DNA into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Biological methods to introduce the DNA of interest into a host cell include the use of DNA and RNA viral vectors. The main advantage of physical methods is that they are not associated with pathological or oncogenic processes of viruses. However, they are less precise, often resulting in multiple copy insertions, random integration, disruption of foreign and endogenous gene sequences, and unpredictable expression.

As used herein, the term "host cell" includes cell lines, primary cells and prokaryotic cells. "Cell line" is intended to refer to well-characterized homogenous, biologically pure populations of cells. These cells may be eukaryotic cells that are neoplastic or which have been "immortalized" *in vitro* by methods known in the art, or prokaryotic cells. The host cell is preferably of mammalian origin, but host cells of non-mammalian origin are also envisioned. Preferred primary cells are bone marrow cells, e.g., cells including mesenchymal precursor cells, preferably those of humans.

"Genetically altered" is used herein to include any host cell, the genome of which has been altered or augmented, e.g., by viral transduction, transfection or other transformation techniques known to the art, by the presence of at least one DNA sequence, which DNA is also referred to in the art of genetic engineering as "heterologous DNA," "recombinant DNA," "exogenous DNA," "genetically engineered," "non-native," or "foreign DNA". The host cells of the present invention may be produced by transfection with a DNA sequence in a plasmid expression vector, a viral expression vector, or as an isolated linear DNA sequence, or by infection with a recombinant virus, i.e., virus comprising recombinant nucleic acid.

Thus, using an appropriate host-vector system, osteoprotegerin is produced recombinantly by culturing a host cell that is genetically altered with an expression vector containing a nucleic acid sequence encoding osteoprotegerin under conditions such that osteoprotegerin is produced. Osteoprotegerin is produced in the supernatant of genetically altered mammalian host cells or in inclusion bodies of genetically altered bacterial host cells. Osteoprotegerin so produced may be purified by procedures known to one skilled in the art. It is anticipated that the exemplified plasmids and host cells described are for illustrative purposes and that other available plasmids and host cells could also be used.

To confirm the presence of the recombinant DNA in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence of a polypeptide expressed from a gene present in the vector, e.g., by immunological means (immunoprecipitations, immunoaffinity columns, ELISAs and Western blots) or by any other assay useful to identify molecules falling within the scope of the invention.

To detect and quantitate RNA produced from introduced DNA segments, RT-PCR may be employed. In this application of PCR, it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of

the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique demonstrates the presence of an RNA species and gives information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the DNA segment in question, they do not provide information as to whether the DNA segment is being expressed. Expression may be evaluated by specifically identifying the polypeptide products of the introduced DNA sequences or evaluating the phenotypic changes brought about by the expression of the introduced DNA segment in the host cell.

Assays to Detect Osteoprotegerin Activity

An *in vitro* culture model of osteoclast formation (osteoclast forming assay) (Udagawa et al., 1989; Udagawa et al., 1990) may be employed to detect osteoprotegerin activity, e.g., in cells transduced with a viral vector encoding osteoprotegerin. The culture system employs a combination of bone marrow cells and cells from bone marrow stromal cell lines. A description of a modification of this culture system has been described in Lacey et al. (1995). Bone marrow cells, flushed from the femurs and tibiae of mice, are cultured overnight in culture media (alpha MEM with 10% heat inactivated fetal bovine serum) supplemented with 500 U/ml CSF-1 (colony stimulating factor 1, also called M-CSF), a hematopoietic growth factor specific for cells of the monocyte/macrophage family lineage. Following this incubation, the non-adherent cells are collected, subjected to gradient purification, and then cocultured with cells from the bone marrow cell line ST2 (1×10^6 non-adherent cells: 1×10^5 ST2 cells/ml media). The media is supplemented with dexamethasone (100 nM) and the biologically-active metabolite of vitamin D3 known as 1, 25 dihydroxyvitamin D3 (1,25 (OH) 2 D3, 10 nM). To enhance osteoclast appearance, prostaglandin E2 (250 nM) is added to some cultures. The coculture period usually ranges from 8-10 days and the media, with all of the supplements freshly added, is renewed every 3-4 days. At various intervals, the cultures are assessed for the presence of tartrate acid phosphatase (TRAP)

using either a histochemical stain (Sigma Kit # 387A, Sigma, St. Louis, MO) or a TRAP solution assay. The TRAP histochemical method allows for the identification of osteoclasts phenotypically which are multinucleated (≥ 3 nuclei) cells that are also TRAP+. The solution assay involves lysing the osteoclast-containing cultures in a citrate buffer (100 mM, pH 5.0) containing 0.1% Triton X-100. Tartrate resistant acid phosphatase activity is then measured based on the conversion of *p*-nitrophenylphosphate (20 nM) to *p*-nitrophenol in the presence of 80 mM sodium tartrate which occurs during a 3-5 minute incubation at room temperature. The reaction is terminated by the addition of NaOH to a final concentration of 0.5 M. The optical density at 405 nm is measured and the results are plotted.

The method described by Lacey et al. (1995) employs bone marrow macrophages as osteoclast precursors. The osteoclast precursors are derived by taking the nonadherent bone marrow cells after an overnight incubation in CSF-1/M-CSF, and culturing the cells for an additional 4 days with 1,000 - 2,000 U/ml CSF-1. Following 4 days of culture, termed the growth phase, the non-adherent cells are removed. The adherent cells, which are bone marrow macrophages, can then be exposed for up to 2 days to various treatments in the presence of 1,000 - 2,000 U/m1 CSF-1. This 2 day period is called the intermediate differentiation period. Thereafter, the cell layers are again rinsed and then ST-2 cells (1 X 10^5 cell/ml), dexamethasone (100 nM) and 1,25 (OH) 2 D3 (10 nM) are added for the last 8 days for what is termed the terminal differentiation period. Test agents can be added during this terminal period as well. Acquisition of phenotypic markers of osteoclast differentiation are acquired during this terminal period.

Other methods to detect biologically active osteoprotegerin are known, and include immunoassays such as enzyme-linked immunosorbent assays (ELISAs) which permit the quantification of osteoprotegerin concentrations (Amgen, Thousand Oaks, CA). *In vivo* methods include the use of animal models for a human disease. For example, inoculation of ARH-77 cells into immunodeficient mice, e.g., SCID mice, yields a murine model for human myeloma (Alsina et al., 1996; Huang et al., 1993), inoculation of a human breast cancer cell line, MDA-MB-231 cells (American Type Culture Collection, HTB-

26), into immunodeficient mice, e.g., nude mice, results in osteolytic bone metastases characteristic of those observed in breast cancer patients (Guise et al., 1996), and inoculation of B16-G3.26 melanoma cells into Sl/Sl^{d}, W/W^{v} , and congenic +/+ mice results in multi-tissue metastases, including bone and bone marrow metastases (Arguello et al., 1992). Thus, ARH-77 cells, MDA-MB-231 cells or B16-G3.26 melanoma cells can be transfected or transduced with a nucleic acid molecule encoding osteoprotegerin and the transfected or transduced cells introduced into an appropriate host organism, e.g., immunocompromised mice. The phenotype of mice transplanted with transfected or transduced cells is compared to mice transplanted with control cells, e.g., Ca^{2+} concentration in whole blood, radiographs and osteolytic lesion area, bone histology and histomorphometry (see Guise et al., 1996; Yin et al., 1999) may be assessed to determine whether the expression of osteoprotegerin inhibits, prevents or reverses osteolytic lesion formation or development.

Formulations and Routes of Administration for the Compositions and Genetically Altered Cells of the Invention

The invention also provides for compositions comprising a nucleic acid molecule of the invention, e.g., in a liposome, a recombinant virus or isolated nucleic acid, together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. Also provided are cells that are genetically altered with the nucleic acid molecule of the invention. The compositions and/or genetically altered cells are suitable for the delivery of part or all of the osteoprotegerin coding region to an organism, preferably a mammal such as a human, e.g., as part of a gene therapy regimen. Both systemic and local administration, e.g., intralesional administration such as to a bony or tumor lesion or to the bone marrow space, are envisioned.

Thus, the administration of a nucleic acid molecule may be accomplished through the introduction of cells genetically altered with the nucleic acid molecule (see, for example, Allay et al., 1997; Hou et al., 1999; WO 93/02556), the administration of the nucleic acid molecule itself (see, for example, Felgner et al., U.S. Patent No. 5,580,859, Pardoll et al. (1995); Stevenson et al. (1995); Molling (1997); Donnelly et al. (1995); Yang et al. (1996); Abdallah et al. (1995)), through infection with a recombinant virus or via liposomes.

Pharmaceutical formulations, dosages and routes of administration for nucleic acids are generally disclosed, for example, in Felgner et al., *supra*.

Administration of the nucleic acid molecule, e.g., a composition comprising such a molecule or a cell genetically altered with such a molecule, in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration may be essentially continuous over a period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated. The amount administered is preferably a "therapeutically effective amount", which means an amount which provides a therapeutic effect for a specified condition and route of administration.

One or more suitable unit dosage forms comprising the compositions or genetically altered cells of the invention, can be administered by a variety of routes including oral (e.g., oral delivery of virus), or parenteral, including by rectal, transdermal (e.g., transdermal administration of virus), subcutaneous, (e.g., via a collagen leased implant) intravenous, intramuscular, intraperitoneal, intrathoracic, intraarterial, intracardiac, intralesional, intrapulmonary and intranasal routes. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the nucleic acid molecule, recombinant virus or genetically altered cell with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

The nucleic acid molecule of the invention can also be formulated as elixirs or solutions for convenient oral administration, or the nucleic acid molecule, recombinant virus or genetically altered cell as a solution appropriate for parenteral administration, for instance by intramuscular, subcutaneous (e.g., via a collagen-based implant) or intravenous routes. The pharmaceutical formulations can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension.

Thus, the nucleic acid molecule, recombinant virus or genetically altered cell may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The active ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable vehicles and adjuvants which are well known in the prior art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol", polyglycols and polyethylene glycols, C_1-C_4 alkyl esters of short-chain acids, preferably ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol", isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

The formulations according to the invention can also contain thickening agents such as cellulose and/or cellulose derivatives. They can also contain gums such as xanthan, guar or carbo gum or gum arabic, or alternatively polyethylene glycols, bentones and montmorillonites, and the like.

It is possible to add, if necessary, an adjuvant chosen from antioxidants, surfactants, other preservatives, film-forming, keratolytic or comedolytic agents, perfumes and colorings. Also, other active ingredients may be added, whether for the conditions described or some other condition.

For example, among antioxidants, t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol and its derivatives may be mentioned. The galenical forms chiefly conditioned for topical application take the form of creams, milks, gels, dispersion or microemulsions, lotions thickened to a greater or lesser extent, impregnated pads, ointments or

sticks, or alternatively the form of aerosol formulations in spray or foam form or alternatively in the form of a cake of soap.

For administration to the upper (nasal) or lower respiratory tract by inhalation, the nucleic acid molecule, recombinant virus or genetically altered cell of the invention is conveniently delivered from an insufflator, nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the nucleic acid molecule of the invention may take the form of a dry powder, for example, a powder mix of the therapeutic agent and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, e.g., gelatine or blister packs from which the powder may be administered with the aid of an inhalator, insufflator or a metered-dose inhaler.

For intra-nasal administration, the nucleic acid molecule, recombinant virus or genetically altered cell of the invention may be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker).

Drops, such as eye drops or nose drops, may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents or suspending agents. Liquid sprays are conveniently delivered from pressurized packs. Drops can be delivered via a simple eye dropper-capped bottle, or via a plastic bottle adapted to deliver liquid contents dropwise, via a specially shaped closure.

The nucleic acid molecule of the invention may further be formulated for topical administration in the mouth or throat. For example, the active ingredients may be formulated as a lozenge further comprising a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the composition in

an inert base such as gelatin and glycerin or sucrose and acacia; and mouthwashes comprising the composition of the present invention in a suitable liquid carrier.

The formulations and compositions described herein may also contain other ingredients such as antimicrobial agents, or preservatives. Furthermore, the active ingredients may also be used in combination with other therapeutic agents, for example, bronchodilators.

For purposes of intramuscular injection, solutions in an adjuvant such as sesame or peanut oil or in aqueous propylene glycol can be employed, as well as sterile aqueous solutions. Such aqueous solutions can be buffered, if desired, and the liquid diluent first rendered isotonic with saline or glucose. Solutions of the vector as a free acid (DNA contains acidic phosphate groups) or a pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. A dispersion of viral particles can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In this connection, the sterile aqueous media employed are all readily obtainable by standard techniques well-known to those skilled in the art.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and

the like. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the nucleic acid molecule, recombinant virus or genetically altered cell in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique which yield a powder of the active ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

For purposes of topical administration, dilute sterile, aqueous solutions (usually in about 0.1% to 5% concentration), otherwise similar to the above parenteral solutions, are prepared in containers suitable for incorporation into a transdermal patch, and can include known carriers, such as pharmaceutical grade dimethylsulfoxide (DMSO).

The dosage of the nucleic acid molecule (e.g., between 0.1 μg to 10 mg, preferably between 1 μg to 1 mg), recombinant virus (e.g., between 10^4 to 10^{14} IU/ml, preferably between 10^5 IU/ml to 10^{14} IU/ml), or genetically altered cells (e.g., between about 10^4 to 10^{10}, preferably about 10^5 to 10^9, cells/kg) of this invention which will be most suitable for prophylaxis or treatment will vary with the form of administration, the particular nucleic acid molecule, recombinant virus or genetically altered cell chosen and the physiological characteristics of the particular patient under treatment, which is well within the skill of the art. A more extensive survey of components suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1980).

Methods of Treatment

Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorus), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, osteocytes, osteoblasts and osteoclasts, are involved in the dynamic process by which bone is continually formed and resorbed. Osteoblasts promote formation of bone tissue whereas osteoclasts are associated with resorption. Resorption, or the dissolution of bone matrix and mineral, is a fast and efficient process compared to bone formation and can release large amounts of mineral from bone. Osteoclasts are involved in the regulation of the normal remodeling of skeletal tissue and in resorption induced by hormones. For instance, resorption is stimulated by the secretion of parathyroid hormone in response to decreasing concentrations of calcium ion in extracellular fluids. In contrast, inhibition of resorption is the principal function of calcitonin. In addition, metabolites of vitamin D alter the responsiveness of bone to parathyroid hormone and calcitonin.

After skeletal maturity, the amount of bone in the skeleton reflects the balance (or imbalance) of bone formation and bone resorption. Peak bone mass occurs after skeletal maturity prior to the fourth decade. Between the fourth and fifth decades, the equilibrium shifts and bone resorption dominates. The inevitable decrease in bone mass with advancing years starts earlier in females than males and is distinctly accelerated after menopause in some females (principally those of Caucasian and Asian descent).

Osteopenia is a condition relating generally to any decrease in bone mass to below normal levels. Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in bone resorption with a normal or reduced rate of bone formation. About 25 to 30 percent of all white females in the United States develop symptomatic osteoporosis. A direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric fracture in women 45

years and older. Elderly males also develop symptomatic osteoporosis between the ages of 50 and 70.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been identified which may contribute to the condition. They include alteration in hormone levels accompanying aging and negative calcium balance attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary calcium and vitamin D supplement in an attempt to retard the process. To date, however, an effective treatment for bone loss does not exist.

The invention provides for a method of treating various bone disorders or conditions. The bone disorder or condition may be any disorder or condition characterized by a net bone loss (osteopenia or osteolysis), e.g., due to altered osteoclastogenesis. In general, treatment with osteoprotegerin is anticipated when it is necessary to suppress the rate of bone resorption. Thus, treatment may be done to reduce the rate of bone resorption where the resorption rate is above normal or to reduce bone resorption to below normal levels in order to compensate for below normal levels of bone formation.

Conditions which are associated with altered or aberrant (e.g., increased) osteoclastogenesis include the following: osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathryoidism, Cushing's syndrome, and acromegaly), and osteoporosis due to immobilization; Paget's disease of bone (osteitis deformans) in adults and juveniles; osteomyelitis, or an infectious lesion in bone, leading to bone loss; hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignancies (multiple myeloma, lymphoma and leukemia), idiopathic hypercalcemia, and hypercalcemia associated with hyperthyroidism and renal function disorders; osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic hepatic and renal diseases; osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus and other conditions; bone loss due to rheumatoid arthritis; periodontal bone loss; and osteolytic metastasis.

It is understood that the compositions and genetically altered cells of the invention may be used alone, in combination or in conjunction with other factors for the treatment of bone disorders, e.g., factors including but are not limited to the bone morphogenic factors designated BMP-1 through BMP-12, transforming growth factor-β (TGF-β) and TGF-β family members, interleukin-1 inhibitors, TNFα inhibitors, parathyroid hormone and analogs thereof, parathyroid hormone related protein and analogs thereof, E series prostaglandins, bisphosphonates (such as alendronate and others), and bone-enhancing minerals such as fluoride.

The invention will be described by the following non-limiting examples.

Example 1

Preparation of Osteoprotegerin Vectors

E. coli transformed with the pGEM-T-easy-osteoprotegerin plasmid (kindly provided by Dr. Lorenz C. Hofbauer) was grown overnight in a 1 liter volume of LB medium with amplicillin 100 μg/ml at 37°C with rotation. The pGEM-T-easy-osteoprotegerin plasmid was then isolated and purified by alkaline lysis using the Qiagen (Valencia, CA) maxi prep plasmid DNA extraction kit. A 5 μg quantity of pGEM-T-easy-Osteoprotegerin was digested with the restriction endonuclease EcoRI at 37°C for 1 hour, and the digestion product was isolated by electrophoresis on 1.5% agarose gel. The osteoprotegerin band was extracted and purified using the Qiagen DNA gel purification kit, following the manufacturer's protocol.

A plasmid and a retroviral vector were prepared in the following way. pCI.tTA.neo.mGM-CSF (henceforth referred to as pCI) (kindly provided by S. Aga-Mohammadi) and pHR'-CMV.LacZ (henceforth referred to as pHR') (a generous gift from D. Trono) were chosen as the plasmid and retroviral vectors, respectively. A 3 μg quantity of pCI was digested with BamHI at 37°C for 1 hour, yielding the linearized pCI backbone and the mGM-CSF insert, which is flanked by two BamHI restriction sites. A 3 μg quantity of pHR' was digested simultaneously with BamHI and XhoI at 37°C for 1 hour, yielding the linearized pHR' backbone and the LacZ insert, which is flanked by a BamHI site at the 5' terminus and by a XhoI site at the 3' terminus. The products of both digestion reactions were run on 0.7% agarose gel. The linearized pCI and pHR', which

localized to their expected size positions, were extracted, and purified from the gel using the Qiagen DNA gel purification kit.

Because of incompatible restriction site termini, the osteoprotegerin gene was amplified by polymerase chain reaction (PCR), using 5' and 3' primers containing two tandem Bam HI sites and two tandem XhoI sites (5' Bam HI: GGATCCGGATCCGTATATATAACGTGATGAGC (SEQ ID NO:1); 3' Bam HI: GGATCCGGATCCTTATCATCCATGGGATCTCG (SEQ ID NO:2); 5' XhoI: CTCGAGCTCGAGGTATATATAACGTGATGAGC (SEQ ID NO:3); 3' XhoI: CTCGAGCTCGAGTTATCATCCATGGGATCTCG (SEQ ID NO:4)). For the pCI construct, Bam HI sites were added at the 5' and 3' termini. For the pHR' construct, a 5' BamHI site and a 3' XhoI site were added for the sense construct. For the antisense construct, a 5' XhoI site and 3' Bam HI site were added. pCI and pHR'backbones were treated with 1 μg of alkaline phosphatase (New England Biolabs, Beverly MA) for 1 hour, in order to prevent self-ligation and recircularization. The enzymes used at each reaction were heat-inactivated between each step. The pCI backbone and osteoprotegerin insert were then combined in solution in a 3-to-1 molar ratio in favor of the insert. A similar solution was prepared with the pHR' backbone and the osteoprotegerin insert. DNA recombination was performed with the DNA rapid ligation kit (Boehringer, Indianapolis, IN) at room temperature over 5 minutes.

The PCI-osteoprotegerin and pHR'-osteoprotegerin recombinant vectors were then used to transform supercompetent Sure2 *E. coli* (Stratagene, San Diego, CA), following the manufacturer's protocol. The transformed cells were selected through growth on ampicillin-containing LB-agar plates. The resulting colonies were grown and screened for the pCI-osteoprotegerin and pHR'-osteoprotegerin recombinant plasmids using restriction sites unique and asymmetrical within the backbones and osteoprotegerin insert: for pCI-osteoprotegerin, Bsu36I for presence of osteoprotegerin, and BstEII for orientation of the insert; for pHR'-osteoprotegerin, BstEII for presence of osteoprotegerin, and KpnI for its orientation. Using calcium phosphate DNA precipitation, 293 T cells were then transfected with the pHR'-osteoprotegerin construct, thus producing replication-incompetent retroviral vectors.

An adenoviral vector for osteoprotegerin was synthesized in the following way. The pGEM-T-easy-osteoprotegerin parent construct was digested with EcoRI, and the osteoprotegerin insert was isolated and purified after gel electrophoresis as described above. The osteoprotegerin sequence was then amplified by PCR, using 5' and 3' primers both containing two tandem BamHI restriction sites. The amplified osteoprotegerin product with added BamHI sites at both termini was then ligated into the pCR2.1 TA backbone (Invitrogen, Carlsbad, CA) in a 1-to-1 insert-to-backbone ratio. The ligation products were then used to transform Inv2-αF' competent *E. coli* (Invitrogen), following the manufacturer's protocol. The transformed cells were then color-selected through growth on X-gal-coated, ampicillin-containing LB-agar plates. The resulting colonies were grown and screened for the PCR2.1-osteoprotegerin recombinant plasmids using restriction sites unique and asymmetrical within the backbone and osteoprotegerin insert, using Bsu36I for the presence of osteoprotegerin. Osteoprotegerin DNA was subsequently digested from the pCR2.1 backbone using BamHI endonuclease. The osteoprotegerin insert was then ligated into the shuttle vector of the pQBI-AdCMV5-adenovirus. The orientation within the shuttle vector was determined using orientation cutting with AatI. The shuttle vector and adenovirus backbones were then packaged and grown in 293 T cells and functional clones were localized by the presence of cell lysis. The resulting vector consists of the osteoprotegerin insert within a replication-incompetent adenovirus.

The sequence of the osteoprotegerin insert in all 3 recombinant constructs was confirmed (Simonet et al., 1997). Corresponding osteoprotegerin antisense vectors were also prepared as negative controls.

Example 2

In Vivo Murine Model for Human Multiple Myeloma

Materials and Methods

Cell Line

The human myeloma cell line ARH-77 is obtained from the American Type Culture Collection (Rockville, MD). Cells are maintained in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 100 μM L-glutamine, 10% fetal bovine serum, 100 μg/ml of penicillin, and 100 μg/ml of streptomycin. Prior to

inoculation into mice, ARH-77 cells are washed and suspended at an appropriate concentration in sterile PBS. Conditioned media from the cultures is collected and concentrated 4X using a Microconcentrator Centriprep 3 (Amicon, Danvers, MA).

Mice

SCID (C.B.-17 SCID/SCID) mice, 6 to 8 wk of age, are obtained from a specific-pathogen-free colony.

Irradiation and Inoculation

Recipient mice are exposed to 150-250 cGy (rads) of radiation from a γ source. Cells are inoculated into mice 24 hours after total-body irradiation. Irradiated mice are given a single i.v. injection of 0.1 to 10 x 10^6 cells in the tail vein.

Mice are then followed-up weekly by measurement of serum calcium levels and whole body x-rays. When the control ARH-77 mice become hypercalcemic (whole blood Ca^{++}>1.35 mmol/L), all mice are anesthetized with methoxyflurane (Pitman Moore, Mundelein, IL) and killed by cervical dislocation. Marrow cell and marrow plasma were then isolated from long bones. Vertebral bones are dissected free of surrounding tissue and used for bone histomorphometry studies.

Histopathology

Animals are sacrificed, and excised tissues are fixed in 10% buffered formalin, embedded in paraffin sectioned, and stained with hematoxylin:eosin prior to routine histopathological examination.

Bones from all animals are fixed in 10% phosphate-buffered formalin for 24 to 48 hours, decalcified in 14% EDTA for 2 to 3 weeks, processed through graded alcohols, and embedded in paraffin wax. Serial sections (3 μm thick) of vertebral bodies are cut at various levels and stained with hematoxylin, eosin, orange G, and phloxine for histologic analysis. Consecutive sections (2 μm thick) are also taken at various levels to allow the examination of the same cell for expression of TRAP, a marker enzyme of osteoclasts. These sections are deparaffinized in xylene and immersed in acetone for 5 minutes. They are then placed for 1 hour in the substrate solution that contained 0.09 mmol/L naphthol-AS-BI phosphate (Sigma) in 0.2 mol/L acetate and 0.4 mol/L L-(+)-tartaric acid

(Sigma) at pH 4.9. Sections are then placed for 30 minutes in hexazotized pararosaniline in 0.2 mol/L acetate buffer with 0.4 mol/L tartaric acid, rinsed, and counterstained with methyl green and light green SF yellow (Sigma).

For blood smears, the air-dried peripheral blood smears from SCID mice are stained with a Baxter staining kit (Baxter, Miami, FL). Briefly, smears are fixed with Diff-Quik fixative (1.8 mg/liter of triarylmethane dye:100% PDC in methyl alcohol) for 5 seconds, air dried for 5 minutes, and then stained with Diff-Quik Solution I (1 g/liter of xanthene dye:100% PDC:buffer:0.01% NaN_3) for 5 seconds and Diff-Quik Solution II (1.25 g/liter of triazine dye mixture:100% PDC:0.625 g/liter of azure Ag 0.625 g/liter of methylene blue:buffer) for 5 seconds. Slides are then rinsed in distilled H_2O.

Collection of bone marrow plasma and assay of early osteoclast precursors (colony-forming unit-granulocyte-macrophage [CFU-GM])

Femurs are removed aseptically and dissected free of adhering tissue. The ends of the femurs are cut with a scalpel blade and the marrow is flushed with 5 mL of α-minimal essential medium (α-MEM) containing 0.1% (vol/vol) penicillin-streptomycin (Gibco) using a 25-gauge needle. The cell suspension is centrifuged at 400 g for 10 minutes and bone marrow plasma is collected and concentrated 5X using a Microconcentrator (Amicon, Danvers, MA).

Bone marrow cells (5 x 10^6/mL) are resuspended in α-MEM containing 15% FCS (Hyclone Laboratory) and incubated for 2 hours at 37°C in a humid atmosphere of 5% CO_2-air to remove cells adherent to plastic. The nonadherent bone marrow cells (10^5/mL) are plated on 35-mm tissue culture dishes (Falcon, Lincoln Park, NJ) in 1 mL of 0.8% methylcellulose (Aldrich co., Milwaukee, WI), supplemented with 20% FCS, 1% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO), and 1.25 ng/mL of recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF; Immunex Co., Seattle, WA) as the source of colony-stimulating activity. Each assay is performed in triplicate. Cultures are incubated at 37°C in a humid atmosphere of 5% CO_2-air for 7 days, at which time colonies (>40 cells) and clusters (>10 and <40 cells) are counted with an inverted microscope.

Bone resorption assays

Timed-pregnant rats are injected with 250 μCi of $^{45}CaCl_2$ at day 18 of gestation. One day later, the rats are killed by cervical dislocation and the embryos removed. The explanted radii and ulnae are cultured on circles of mixed ester membrane filters (0.45 μm; Whatman, Hillsboro, OR) on stainless steel grids in 0.5 mL of chemically defined medium (Sigma) supplemented with 1 mg/mL BSA (Sigma) and penicillin-streptomycin (50 U/mL and 50 mg/mL, respectively) in 5% CO_2 in air at 37°C, as modified by Raisz and Niemann (1969). The radii and ulnae are incubated for 24 hours in control media to allow for the removal of the exchangeable ^{45}Ca before transferring to equilibrated control or experimental media. Experimental media contains varying concentrations of either bone marrow plasma from osteoprotegerin expressing ARH-77 mice, or control ARH-77 mice, media conditioned by ARH-77 cells *in vitro*, or untreated culture media. Control or experimental media are then changed after 72 hours. The bone explants are incubated for a total of 5 days. Bone-resorbing activity is measured as the percentage of total ^{45}Ca released from the bone into the media over the 5 days of incubation.

Assay of bone-resorbing cytokines

The human and murine cytokines that induce bone resorption (human IL-1β, human TGFα, PTHrP, human IL-6, human TNFβ, murine IL-6, murine TNFβ, and murine IL-1α) are measured in the peripheral blood sera and bone marrow plasma of osteoprotegerin expressing ARH-77, control ARH-77 mice, and the ARH-77 cell conditioned media using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Boston, MA). The lower limit of detection for cytokines in these assays is approximately 10 pg/mL.

Human Immunoglobulin Assays

All assays for human immunoglobulin are preformed by established methods. Levels of human immunoglobulin in the sera of SCID mice with ARH-77 tumors are quantitated by electrophoresis on agarose gels followed by densitometry with a Cliniscan 3 (Helena, Beaumount, TX). Mice are bled after i.v. inoculation when the hind legs become paralyzed. The serum from normal SCID mice serves as a negative control. Concentrations of human IgG are calculated form the standard curve.

For isotyping human immunoglubulin light chains in the sear of SCID mice, two different methods may be employed. One method employs immunofixation (Sevia, Atlanta, GA) to detect the human IgG Kappa produced by ARH-77 cells. The other method employs capillary electrophoresis (Beckman, Brea, CA).

Results

The ARH-77 murine model consists of injecting a human myeloma cell line as a xenograft in severe combined immunodeficient (SCID) mice. The bone loss, spinal compression, shortened survival, and serum monoclonal immunoglobulin peak characterizing this system have made it a favored multiple myeloma model. Moreover, the osteoclast overactivation that characterizes multiple myeloma is the common denominator of several other metabolic bone diseases such as Paget's disease and osteoporosis.

ARH-77 myeloma cells are transduced with recombinant virus comprising osteoprotegerin nucleic acid, and osteoprotegerin concentrations in the conditioned medium are determined, e.g., by Western blot/ELISA and by TRAP-based osteoclast formation cell culture assay. These results are compared to ARH-77 cells transduced with either the antisense osteoprotegerin sequence or with vectors lacking osteoprotegerin, and to untransduced (control) ARH-77 cells. Mice are then injected with either osteoprotegerin expressing ARH-77 cells or with control ARH-77 cells.

Control mice that survive irradiation develop hind limb paralysis 28 to 35 days after the injection of the cells and lose 10% of their lean body mass by the time they become paraplegic. Serum calcium levels, serum bone resorption markers and total body X-ray are used to evaluate the mice prior to sacrifice, e.g., for hypercalcemia, bone resorption and formation of osteolytic lesions. After being sacrificed, mouse bones are studied histomorphometrically to determine the efficacy of osteoprotegerin gene expression in preventing the increased osteoclast numbers and activity in control mice. Marrow plasma is analyzed for cytokine levels and marrow cells for osteoclast colony formation. Marrow plasma is also analyzed using a bone resorption assay. Cytokine levels in the blood and serum are determined.

Control ARH-77 mice develop hypercalcemia approximately 5 days after becoming paraplegic. Multiple lytic lesions and diffuse osteopenia are detected in these hypercalcemic mice by x-rays. Hypercalcemia and lytic bone lesions are reduced or absent in mice transplanted with osteoprotegerin expressing ARH-77 cells relative to control mice.

Bone marrow plasma from ARH-77 mice induces significant bone resorption in the fetal rat long bone resorption assay when compared with bone marrow plasma from osteoprotegerin-transduced ARH-77 mice. Conditioned media from ARH-77 cells induces significant bone resorption in the same assay when compared with untreated media. Antibodies against TNF and lymphotoxin fail to block this effect significantly.

To determine if osteoclast precursors in osteoprotegerin-transduced ARH-77 mice are decreased in marrow relative to control ARH-77 mice, cultures for early osteoclast precursors (CFU-GM) are performed. CFU-GM colonies from osteoprotegerin-transduced ARH-77 mice are decreased relative to control ARH-77 mice.

Histologically, the control ARH-77 mice show infiltration of myeloma cells in the liver, spleen, and bones. The vertebrae and long bones have marked infiltration by the tumor with loss of bony trabeculae and increased osteoclast numbers without an osteoblastic response. Deep resorption pits are associated with the osteoclasts adjacent to myeloma cells. In contrast, the bone next to normal bone marrow has a smooth contour. This increase in osteoclast numbers is even more dramatic when the bone sections are stained for TRAP, a marker enzyme for osteoclasts. There is a marked increase in osteoclast numbers in areas of bone adjacent to myeloma cells, but not in areas of bone adjacent to normal bone marrow. In contrast, in osteoprotegerin expressing ARH-77 mice, little or no infiltration is observed in spleen, liver or bone, and osteoclast numbers are less than those in control ARH-77 mice.

Example 3

Transplant of Osteoprotegerin-Transduced Mesenchymal Progenitors into Humans

Materials and Methods

Bone marrow harvest

BM aspirates (10 ml from two 5 ml aspirations) are obtained from the posterior iliac crest of individuals who had given informed consent. Although a small amount of peripheral blood typically is aspirated along with the marrow-derived cells, the peripheral blood does not contain mesenchymal precursor cells (MPCs).

Preparation and propagation of marrow-derived human MPCs

Preparation of the adherent marrow-derived cells has previously been described (Haynesworth et al., 1992). Briefly, single-cell suspensions of bone marrow are layered on 70% (Sigma, St. Louis, MO) gradients and low-density mononuclear cells are recovered. Fifty $\times 10^6$ cells are plated in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS), prescreened for growth and maintenance of the osteogenic potential of hMPCs, as described in Goshima et al., 1991a and 1991b; Lennon et al., 1995), on 100 mm^2 plastic tissue culture dishes at 37°C, 5% CO_2. After 3 days, the medium is changed to remove nonadherent hematopoietic cells. Thereafter, the medium is changed twice weekly. Approximately 10-12 days after primary culture, the cells are detached from the plate with 0.25% trypsin containing 1 mM EDTA (Gibco) for 5 minutes at 37°C. They are diluted 1:3 and cyclically replated in fresh medium when cells reached 80% confluence.

Retroviral transduction of hMPC

hMPCs are grown in DMEM + 20-30% heat-inactivated (HI) FBS for 18-24 hours following first or second passage to increase cell proliferation and enhance the rate of gene transfer. Medium is replaced with 4 ml of 0.45 μm filtered viral supernatant, containing 6 μg/ml Polybrene (Sigma, St. Louis, MO). After 6 hours, viral supernatant is removed and cells are cultured in DMEM + 30% HI FBS for 18 hours and repeated daily for 4 days. The frequency of infection and gene expression is determined. For all *in vivo* experiments, transduced cell populations, not individual clones, are used.

Transplantation

For transplant between 5-8 x 10^8 cells/kg are employed. For allogeneic transplant, appropriate chemotherapeutic regimes are used. Cells are intravenously infused into each patient.

Bone histologic studies

Patients receive 3-day courses of tetracycline at approximately 3 weeks and 1 week before biopsy. A 5.0 mm core of iliac bone is taken before and 6 months after transplantation with a trephine inserted through a 1.5 cm incision, from patients sedated by general anesthesia. Histologic changes are determined on sections 5 μm in thickness of polymethyl methacrylate-embedded samples, using a Zeiss microscope.

Mesenchymal cell cultures

Osteoblasts from bone biopsies are prepared and maintained in culture as specifically described for this cell type by Robey and Termine (Robey et al., 1985). Bone fragments are dissected from soft tissue, progressively 'minced' to a fine granular consistency, digested with collagenase and placed into culture. Flow cytometric analysis indicates a lack of lymphohemopoietic cells in the osteoblast preparations.

Dual energy X-ray absorptiometry

Measurements of total body bone mineral content are done on a whole-body scanner (Hologic QDR 2000 Densitometer; Hologic, Inc., Waltham, Massachusetts), as described (Koo et al., 1998; Koo et al., 1995a; Koo et al., 1995b).

Results

Patients are intravenously infused with autologous osteoprotegerin-transduced bone marrow. All show engraftment with hemopoietic donor cells. Osteoblasts are cultured from fresh bone biopsy specimens. The individual adherent cells in the cultures have typical osteoblast morphology, express alkaline phosphatase and produce stainable matrix. Flow cytometric analysis of these cells indicates a lack of contaminating lymphohemopoietic cells.

Engraftment of autologous osteoprotegerin-transduced bone marrow is associated with improvements in bone histology. A specimen of trabecular bone taken after transplantation shows a reduced number of osteoclasts, and resorption

lacunae. These findings are consistent with a reduction in osteoclast recruitment and activation. Bone matrix deposition and mineralization in patients engrafted with osteoprotegerin-transduced bone marrow are normal. Overall, there is an increase in the total body bone mineral content, as determined by measurements with dual energy X-ray absorptiometry.

Example 4

Osteoprotegerin Overexpression and Myelomatous Skeletal Disease in SCID Mice Injected with ARH-77 Cells

Methods

Lentiviral Vector Production

Replication-incompetent lentiviruses were produced as follows. On day 1, 293T cells (ATCC) were transfected by calcium phosphate precipitation (Promega) with 3 plasmids: one bearing the lentiviral nucleocapsid genes, one with the VSV.G envelope, and one with the osteoprotegerin transgene downstream from a CMV promoter, flanked by 5' and 3' long terminal repeats (LTRs). The 293T cells were subsequently incubated overnight at 37°C. On day 2, the transfection medium was removed by aspiration and replaced with growth medium (DMEM + 10% fetal calf serum (FCS) + 1% penicillin/streptomycin). The cells were then incubated at 37°C for an additional 48 hours. On day 4, the supernatant was aspirated from the 293T cells, placed in a sterile syringe, and passed through a 0.45 μm filter. This protocol was also used to produce lentiviral vector containing the lacZ transgene, to be used for quantification of infection efficiency.

Infection of ARH-77 Cells with Lentiviral Vector

Human multiple myeloma ARH-77 suspension cells (donated by J. Lust) were cultured in RPMI-1640 + 10% FCS + 1% penicillin/streptomycin, and passaged every 3-4 days at a 1:4 ratio. The cells were passaged 24 hours preceding infection. On the day of infection, the ARH-77 cells were centrifuged at 1,000 rpm for 5 minutes, washed with serum-free RPMI-1640, centrifuged again at the same settings, then resuspended in serum-free RPMI-1640 + polybrene 16 μg/ml, to a final density of 1×10^6 cells per ml. Ten milliliters (1×10^7 cells) were then placed in a T-75 flask, to which 10 ml of the above viral supernatant were then added. Infections were performed in parallel with

osteoprotegerin- and lacZ- bearing vectors. Infected ARH-77 cells were incubated for 12 hours at 37°C, then virus was removed by sequential centrifugation and washing with serum-free RPMI-1640. The cells were resuspended and cultured in growth medium under the above conditions for 72 hours, then passaged 24 hours before injection into SCID mice.

Determination of Transduction Efficiency and Osteoprotegerin Expression

Transduction efficiency was determined by infecting 1 x 10^6 ARH-77 cells with lentiviral vector bearing lacZ as the reporter gene. Transduced cells were cultured at 37°C for 72 hours, then stained for X-gal using a standard staining protocol. Osteoprotegerin expression *in vitro* was determined by infecting 1 x 10^6 ARH-77 cells with lentiviral vector containing the osteoprotegerin transgene. Transduced cells were cultured at 37°C for 72 hours in 1.5 ml of serum-free, phenol-free medium containing 0.125% bovine serum albumin (BSA). The conditioned medium was then harvested and separated from ARH-77 cells by centrifugation. Finally, osteoprotegerin concentration of the conditioned medium was measured by enzyme-linked immunosorbent assay (ELISA, graciously provided by Amgen Corp., Thousand Oaks, CA). Readings were divided by 3 to yield ng/ml per 1 x 10^6 cells per day.

Injection of ARH-77 Cells Into SCID Mice

Five-week-old female C.B.17 SCID mice were obtained and housed according to institutional standards. The experimental protocol was approved by the Mayo Clinic Institutional Animal Care and Use Committee. Twenty four hours before injection, the mice underwent total-body irradiation with 250 rad (0.43 minute exposure to a ^{137}Cs source, 581 rad/min dose rate). On the day of injection, ARH-77 cells were centrifuged and washed once in PBS 1X then resuspended in PBS 1X at a final concentration of 1 x 10^7 cells per ml. The cells were kept on ice until the time of injection. The mice were placed under general anesthesia and immobilized in a supine position. A volume of 0.1 ml (1 x 10^6 cells) was injected in the left ventricle. A total of 20 mice were injected: 10 with wild-type ARH-77 cells (henceforth the control group), 10 with ARH-77 cells transduced by lentiviral vector with the osteoprotegerin transgene (treatment group).

Assessment of Outcome of Experimental Mice

All 20 injected mice were evaluated immediately following intracardiac injection, then twice daily for the following 60-day experimental period. Two negative control mice (having received neither total-body irradiation nor intracardiac injections) of the same litter were used for comparison. Clinical end-points included food intake, spontaneous and stimulated movement, changes in coat, weight loss, hindlimb paraplegia, and death (either spontaneous or euthanasia for humane reasons). Radiologic end-points included plain skeletal x-rays (at time of sacrifice) and dual-energy x-ray absorptiometry (DEXA) using an instrument specifically designed for mouse bone densitometry (PIXImus, Lunar, % CV 0.85). In order to adjust for any increases in bone mineral density (BMD) over time, given that the mice were studied during their natural growth period, every mouse undergoing DEXA was matched with a mouse from the other group. For DEXA analysis of specific bones, the same bones and parameters for the region of interest (ROI) were used in all mice. Small variations in defining the ROI (purposely introduced) did not significantly affect BMD readings. The status of the mice (live or dead, previously frozen/thawed) also did not significantly influence BMD results. All mice were autopsied at time of death, and mice with a large amount of pericardial and/or pleural tumor were considered to have received insufficient tumor cells for systemic dissemination, and were thus excluded from analysis.

Results

Infection of ARH-77 Cells with LacZ Lentiviral Vector

ARH-77 human myeloma cells were infected with lentiviral vectors, then gene transfer efficiency was determined relative to viral dose. This was performed by infecting ARH-77 cells with lentiviral vector containing lacZ as the reporter gene, then by staining the cells for X-gal. Using this approach, the percentage of ARH-77 cells showing blue staining was roughly proportional to the amount of virus used for transduction (data not shown). With the highest vector dose, the percentage of blue-staining ARH-77 cells was 40%.

Infection of ARH-77 Cells with Osteoprotegerin Lentiviral Vector

An identical approach was used with the osteoprotegerin-bearing lentiviral vector. Figure 4 shows the correlation between osteoprotegerin

expression, measured in conditioned medium by ELISA, and osteoprotegerin lentiviral vector dose. The highest dose was subsequently used for transduction of the ARH-77 cells injected in the *in vivo* experiment.

Expression of Osteoprotegerin by Infected ARH-77 Cells Over Time

Aliquots of the wild-type ARH-77 cells injected in the control group mice and of the osteoprotegerin-transduced ARH-77 cells injected in the treatment group mice were cultured in parallel under similar *in vitro* conditions. Osteoprotegerin concentration in conditioned medium was measured serially for each cell type over the entire 60-day *in vivo* experimental period (Figure 5). Except for a transient drop at day 41, osteoprotegerin expression remained constant throughout the study period (4.31-5.74 ng/ml per 1 x 10^6 cells per day). In contrast, osteoprotegerin expression by the untransduced wild-type cells was 0.007-0.011 ng/ml per 1 x 10^6 per day. The mean osteoprotegerin expression by the transduced ARH-77 cells was thus over 500 times that of the wild-type ARH-77 cells, and was maintained at that level *in vitro* during the entire 60-day period, without the use of any selection pressure.

Clinical Outcome of Mice Injected with Wild-Type and Osteoprotegerin-Transduced ARH-77 Cells

The clinical course, autopsy and plain skeletal x-ray findings, and bone DEXA scanning results are summarized in Table 1.

Table 1

Control Group (ARH-77 Wild-Type)

Mouse No.	Infection Comments	Clinical/SAC/Death	Autopsy	X-rays	DEXA Date	Total Body BMD	Femur BMD	Tibia BMD	Vertebrae BMD
1	-	Wasted, scruffy day 23, died day 26.	-		-	-	-	-	
2	-	Wasted, scruffy day 23, SAC day 30	Large pleural mets	Negative	x	0.0389	0.0552	0.0458	0.041
3	-	Wasted, scruffy day 23, died day 27	-	-	-	-	-	-	-
4	-	Early paraplegia, wasted, scruffy day 23, died day 24	-	-	-	-	-	-	-
5	-	Wasted, scruffy day 23, SAC day 30	No tumor	Small lytic lesions R prox tibia	x	0.0394	0.0568	0.0459	0.0437
6	-	Wasted, scruffy day 40, SAC day 44	Large tumor	Negative	x + 13	0.0447	0.0676	0.0531	0.0505
7	-	Scruffy day 39, died day 42	No tumor	Negative	x + 14	0.0387	0.0615	0.0465	0.0394
8	-	Wasted and scruffy day 35, early paraplegia 40, died day 41	No tumor	Negative	x + 11	0.0403	0.0604	0.0457	0.0458
9	-	Anesthesia OD, premature death day 15	-	-	-	-	-	-	-
10	-	Slight wasting and scruffiness day 29, died day 30	Minimal pleural mets	Negative	x	0.0397	0.0566	0.0443	0.0426

Treatment Group (ARH-77/Osteoprotegerin Transduction)

Mouse No.	Infection Comments	Clinical/BAC/Death	Autopsy	X-rays	DEXA Data	Total Body BMD	Femur BMD	Tibia BMD	Vertebrae BMD
1	-	Slightly scruffy day 44, wasting day 50.	No tumor	Negative	x	0.0427	0.0637	0.0505	0.0503
2	-	Slightly scruffy day 44, SOB & wasting day 49, SAC day 52.	No tumor	Negative	x	0.0453	0.0662	0.0546	0.0542
3	-	Scruffy day 44, wasting day 52	No tumor	Negative	x + 13	0.0429	0.0619	0.0516	0.0486
4	-	SOB without other Sxs day 35, died day 35	Very large tumor, heart adhered to chest wall	Negative	x + 4	0.0426	0.0617	0.491	0.0482
5	-	Slightly scruffy day 44, SOB day 49, SAC day 51	Moderate pleural tumor	Negative	x + 13	0.0424	0.0632	0.0495	0.0485
6	-	Scruffy and wasted day 31, Partial paraplegia day 37, SAC day 42	No tumor	Negative	x + 11	0.0463	0.0633	0.049	0.0462
7	-	Scruffy day 42, SOB day 49, SAC day 50	Small pleural mets	Negative	x + 13	0.0434	0.0629	0.0493	0.0474
8	Died at injection	-	-	-	-	-	-	-	-
9	-	Anesthesia OD, premature death day 15	-	-	-	-	-	-	-
10	-	Scruffiness, partial paraplegia day 37, SAC day 42	No tumor	Negative	x + 11	0.044	0.0636	0.048	0.0455

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Animals excluded from the analysis include treatment group mouse #8 (treatment 8), which died from technical complications at the time of intracardiac injection of tumor cells, as well as treatment 9 and control 9, which both died prematurely at day 15 of an anesthesia overdose at the time of blood draw. Also excluded were controls 2 and 6 and treatment 4, because of large thoracic tumor and the consequent likelihood of not having received sufficient tumor cells for systemic dissemination. Autopsy, x-ray, or DEXA results are not available for controls 1, 3 and 4, however, those 3 animals showed typical signs of disseminated myeloma and none of the dyspnea characterizing animals dying of extra cardiac tumor. The time frame for death in these 3 mice was also typical for disseminated myeloma. Therefore, controls 1, 3 and 4 were assumed to have died of disseminated disease and were thus included as such in the mortality analyses. Figure 6 compares the survival rates of the control and treatment groups ($p<0.05$ at day 35); at the time of study completion, 2 treatment mice (1 and 3) were showing weight loss but were still alive and active. Unless otherwise specified (e.g., paraplegia), mice were sacrificed (SAC) due to severe emaciation and reduced activity resulting in inability to feed, likely the results of overwhelming tumor burden. Hypercalcemia was not detected (results not shown).

Myelomatous Skeletal Disease in Mice Injected with Wild-Type and Osteoprotegerin-Transduced ARH-77 Cells

The ARH-77 murine model has yielded radiographically detectable osteolytic lesions in only a small percentage of injected animals. In the present cohort, only one of the control mice (5) showed such lesions, in the right proximal tibia. None of the treatment mice showed radiographic evidence of focal osteolysis.

Nevertheless, diffuse bone loss was documented when BMD was measured by DEXA. Figures 7-10 compare BMDs for the control mice (left-hand column), negative control (uninjected, intact) mice (middle column), and treatment mice (right-hand column). Figure 7 shows total-body BMD, excluding the skull, given the disproportionately high skull BMD in rodents and its overshadowing effects on results from the remainder of the skeleton. The mean increase in total-body BMD was 11.1% in Treatment compared to Control mice

(% CV 0.85 for DEXA scanner used). Similarly, the mean increase in for femur, tibia, and vertebral BMD was 8.0%, 10.5%, and 13.5%, respectively (Figures 8-10).

Discussion

Myeloma cells secrete a host of mediators, including interleukin-1β (IL-1β), which promote differentiation of osteoclast precursors into mature osteoclasts, and which also enhance the latter's bone resorbing activity. These mediators are thought to act in both a paracrine and endocrine fashion, thus accounting for the combination of focal and diffuse bone loss encountered in this condition. In turn, by resorbing bone, osteoclasts release from the bone matrix growth factors that are beneficial to the mitotic activity of myeloma cells, hence resulting in a positive feedback loop between myeloma cells and osteoclasts. The ARH-77 murine model was chosen for osteoprotegerin gene transfer, given how osteoclastic overactivation is simultaneously a critical element of myeloma pathophysiology and an ideal target for osteoprotegerin's therapeutic potential.

Although the rate of occurrence of radiographically-detectable osteolytic lesions was too low to detect a difference between treatment and control groups, diffuse bone loss could be assessed with accuracy using DEXA and was found to be prevented when the injected ARH-77 cells were made to express osteoprotegerin above levels expressed by wild-type ARH-77 cells. For all sites measured, the increase in BMD was well above the scanner's precision margin. Moreover, the greatest gain was noted at the vertebral level. This result was anticipated, given that the vertebrae contain the highest density of both myeloma cells and trabecular bone, for which resorptive changes are usually detected earlier than in the less metabolically active cortical bone found in the extremities. Further characterization of the skeletal status is undertaken by measuring biochemical bone turnover markers in stored serum, and by performing bone scintigraphy.

Survival was also significantly improved ($p<0.05$ at day 35) in the treatment mice compared to controls (100% vs. 30% at day 30, 30% vs. 0% at day 60, $P < 0.05$). Although wild-type and transduced cells had similar mitotic rates *in vitro*, a potential mechanism explaining improved survival includes a reduction in bone resorption and thus in matrix growth factor release, resulting in

a lesser tumor burden. Another explanation might be a reduction in tumor and/or bony processes compressing the cervical spine and leading to respiratory arrest. Of note, 2 mice in each of the study groups experienced paraplegia. Although there may have been a trend towards occurring later in the treatment group (days 37 and 37 versus days 23 and 40), increased osteoprotegerin expression did nevertheless not completely prevent this complication. Histologic studies in previous mice cohorts injected with wild-type ARH-77 have shown complete replacement of bone marrow by myeloma cells, with bulging of the vertebral bodies and consequent compression of the adjacent spinal cord. This pathophysiologic process may occur independently of osteoclastic function, and staining specific for osteoclasts is being performed in order to answer this question. In sum, spinal cord compression was an infrequent, pre-terminal event in these animals, and would likely be treated with more targeted therapeutic modalities such as external beam radiotherapy in the human counterpart of this disease.

Of particular interest was to find a positive bone-preserving effect although only 40% of cells were transduced, and serum osteoprotegerin levels were the same in control and treatment mice. Nevertheless, as few as 1×10^5 wild-type ARH-77 cells were required to obtain the above-described phenotype (data not shown). This suggests that only a fraction of tumor cells need be transduced in order to obtain skeletal protection, and emphasizes osteoprotegerin's potent osteoclast inhibitory effect. Although serum osteoprotegerin concentrations were measured in some of the treatment and control mice, with no apparent difference, the osteoprotegerin levels measured in conditioned medium were several-fold higher than for controls. It may be that local synthesis of osteoprotegerin in the bone microenvironment is enough for osteoclastic inhibition, while not being sufficient to affect systemic levels. Although the *in vitro* osteoprotegerin expression spanned the entire 60-day study period, the duration of *in vivo* osteoprotegerin expression is unknown at this juncture, and could potentially be improved further. Nevertheless, 5 of the treatment animals had repeat DEXA scans performed following various intervals, and BMD was maintained at most sites (results not shown).

Osteoprotegerin gene transfer is also useful for other tumor models, such as human breast carcinoma and malignant melanoma murine models, which are characterized by a greater incidence of radiographically-detectable osteolytic lesions, as well as osteoporosis animal models. For example, osteoprotegerin viral vectors are administered systemically, targeting expression by using a bone-specific transcription promoter. Alternatively, another osteoprotegerin gene transfer approach is the *ex vivo* transduction of bone marrow progenitor cells, using the intrinsic bone marrow tropism of those cells upon systemic reinjection.

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. A method to inhibit or prevent osteoclastic overactivity in a mammal, comprising:

(a) contacting pluripotent mammalian cells with an amount of a composition comprising a nucleic acid molecule encoding osteoprotegerin, a variant thereof, or a biologically active fragment thereof so as to yield genetically altered mammalian cells comprising the nucleic acid molecule; and

(b) introducing the genetically altered cells into the mammal so as to alter osteoclastogenesis in the mammal.

2. The method of claim 1 wherein the mammal is a human.

3. The method of claim 1 wherein the introduction is intravenous, intraarterial, intramuscular, intrathoracic, intraperitoneal, intrapulmonary, or intranasal.

4. The method of claim 1 wherein the cells are introduced in an implantable device.

5. The method of claim 1 wherein the composition comprises recombinant adenovirus comprising the nucleic acid molecule.

6. The method of claim 1 wherein the composition comprises recombinant retrovirus comprising the nucleic acid molecule.

7. The composition of claim 1 wherein the composition comprises recombinant lentivirus comprising the nucleic acid molecule.

8. The composition of claim 1 wherein the composition comprises isolated and purified DNA in an aqueous medium.

9. The method of claim 1 wherein the nucleic acid molecule encodes human osteoprotegerin.

10. The method of claim 1 wherein the mammal has multiple myeloma, osteolytic metastatic lesions, hypercalcemia, involutional osteoporosis, secondary osteopetrosis, Paget's disease, refractory hyperparathyroidism, or osteopenia.

11. The method of claim 1 wherein the mammal has an osteolytic lesion.

12. The method of claim 11 wherein the cells are introduced to the lesion.

13. The method of claim 1 wherein the cells are mesenchymal precursor cells.

14. A method to introduce exogenous nucleic acid into unfractionated bone marrow cells, comprising: contacting unfractionated mammalian bone marrow cells with an amount of a composition comprising a nucleic acid molecule encoding osteoprotegerin, a variant thereof, or a biologically active fragment thereof so as to yield genetically altered mammalian cells.

15. The method of claim 14 wherein the cells are human cells.

16. The method of claim 14 further comprising introducing the genetically altered cells into a mammal.

17. The method of claim 14 wherein the composition comprises recombinant retrovirus comprising the nucleic acid molecule.

18. The method of claim 14 wherein the composition comprises recombinant adenovirus comprising the nucleic acid molecule.

19. The method of claim 14 wherein the composition comprises recombinant lentivirus comprising the nucleic acid molecule.

20. The method of claim 14 wherein the cells are from a mammal having multiple myeloma.

21. A recombinant retrovirus comprising nucleic acid encoding osteoprotegerin.

22. A recombinant adenovirus comprising nucleic acid encoding osteoprotegerin.

23. A recombinant lentivirus comprising nucleic acid encoding osteoprotegerin.

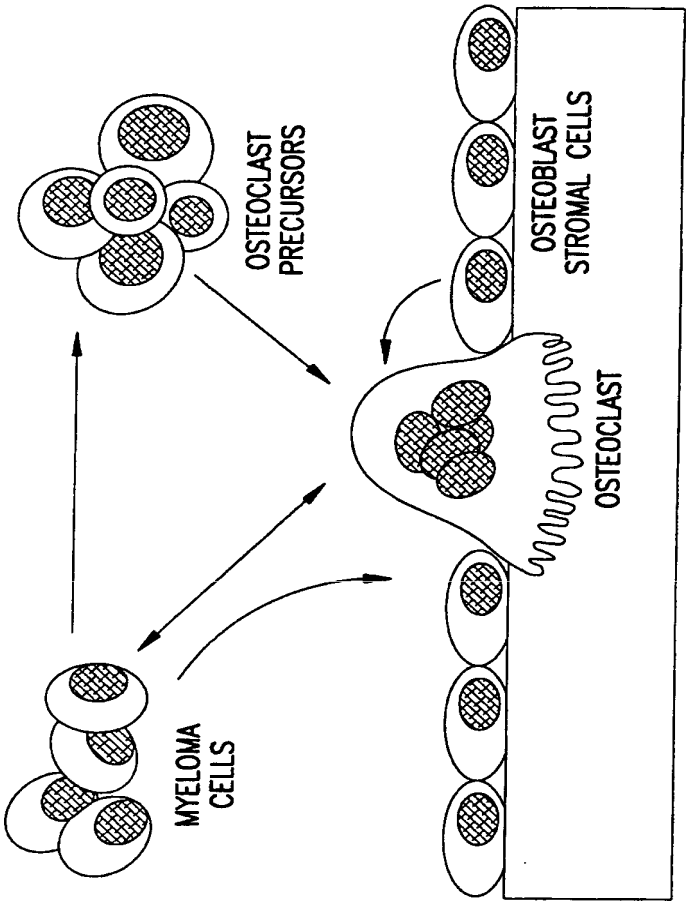

OSTEOCLAST PRECURSORS
OSTEOBLAST STROMAL CELLS
OSTEOCLAST
MYELOMA CELLS

FIG. 1

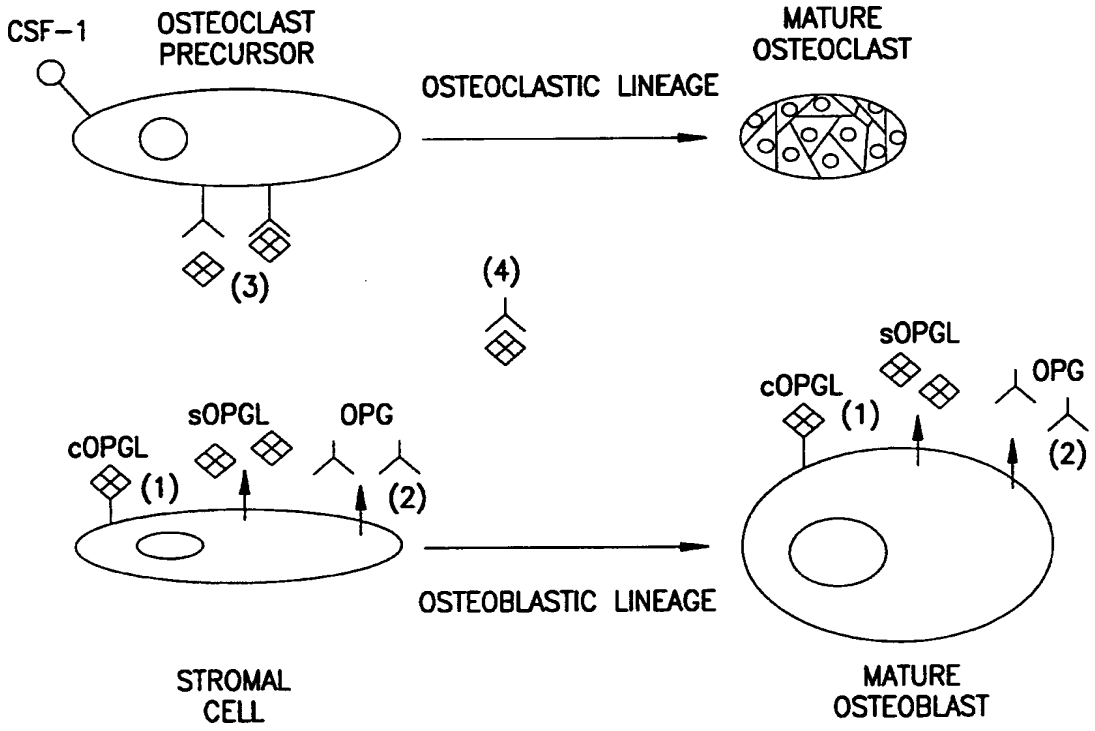
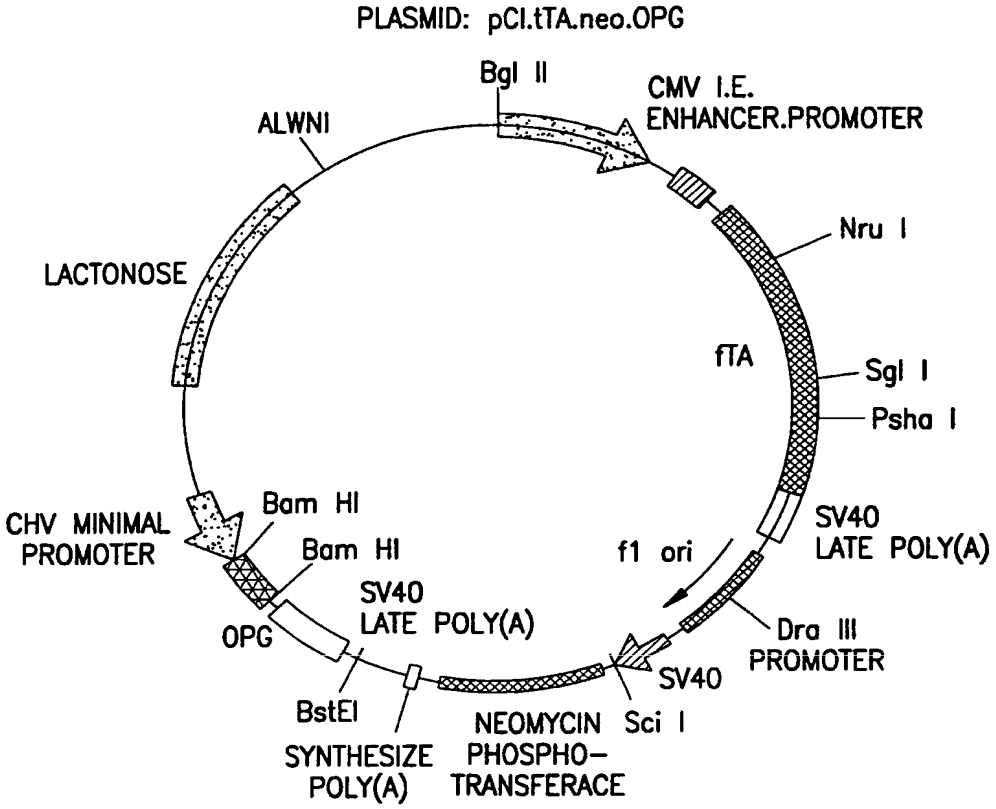

CSF-1
OSTEOCLAST PRECURSOR
OSTEOCLASTIC LINEAGE
MATURE OSTEOCLAST
(3)
(4)
sOPGL
cOPGL
(1)
OPG
(2)
OSTEOBLASTIC LINEAGE
STROMAL CELL
MATURE OSTEOBLAST

FIG. 2


PLASMID: pCI.tTA.neo.OPG
Bgl II
CMV I.E.
ENHANCER.PROMOTER
ALWNI
Nru I
LACTONOSE
fTA
Sgl I
Psha I
SV40
LATE POLY(A)
f1 ori
Dra III
PROMOTER
SV40
Sci I
NEOMYCIN
PHOSPHO-
TRANSFERACE
BstEI
SYNTHESIZE
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OPG
SV40
LATE POLY(A)
Bam HI
Bam HI
CHV MINIMAL
PROMOTER

FIG. 3A

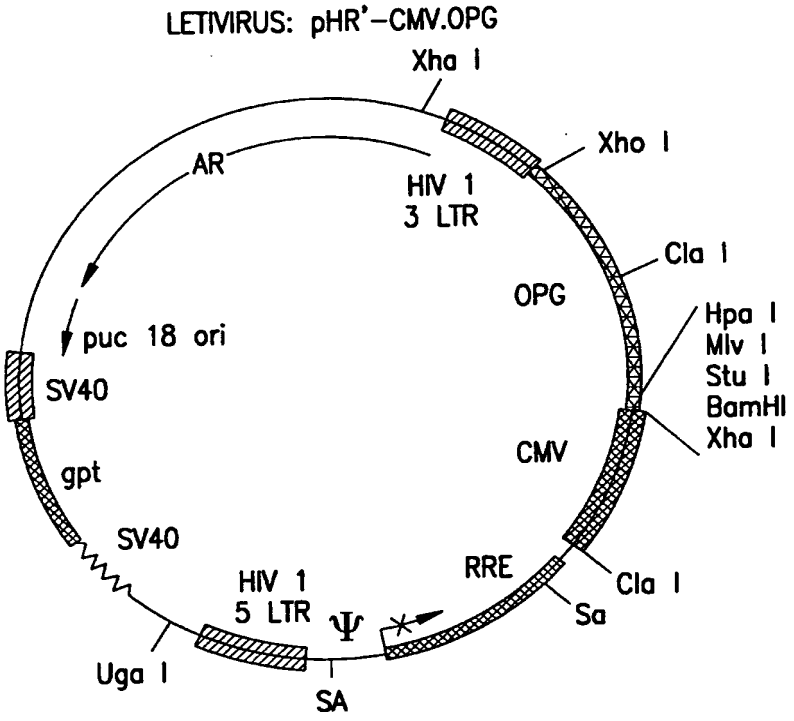
LETIVIRUS: pHR'-CMV.OPG
Xha I
Xho I
AR
HIV 1
3 LTR
Cla I
OPG
Hpa I
Mlv I
Stu I
BamHI
Xha I
puc 18 ori
SV40
CMV
gpt
SV40
RRE
Cla I
HIV 1
5 LTR
Ψ
Sa
Uga I
SA

FIG. 3B

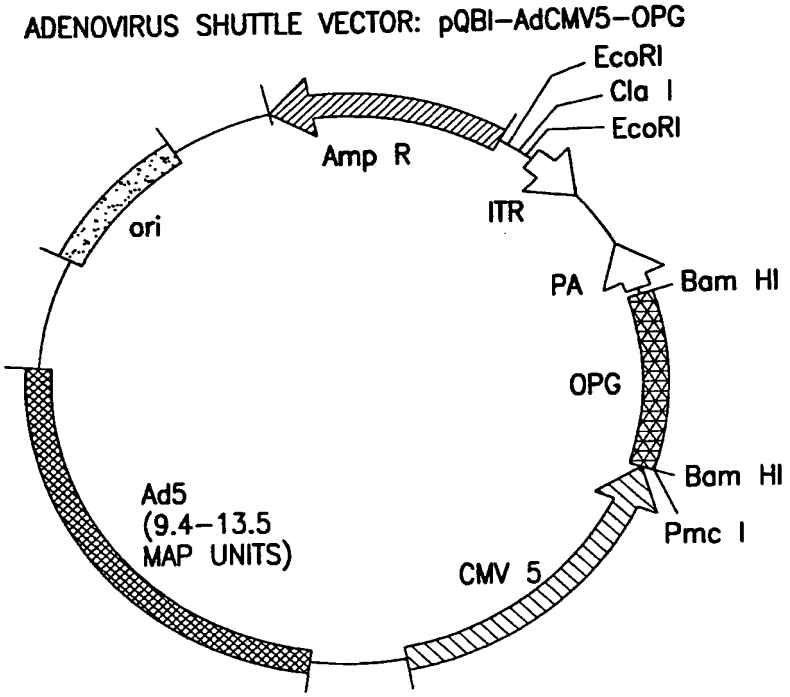
ADENOVIRUS SHUTTLE VECTOR: pQBI-AdCMV5-OPG
EcoRI
Cla I
EcoRI
Amp R
ITR
ori
PA
Bam HI
OPG
Bam HI
Pmc I
Ad5
(9.4-13.5
MAP UNITS)
CMV 5

FIG. 3C

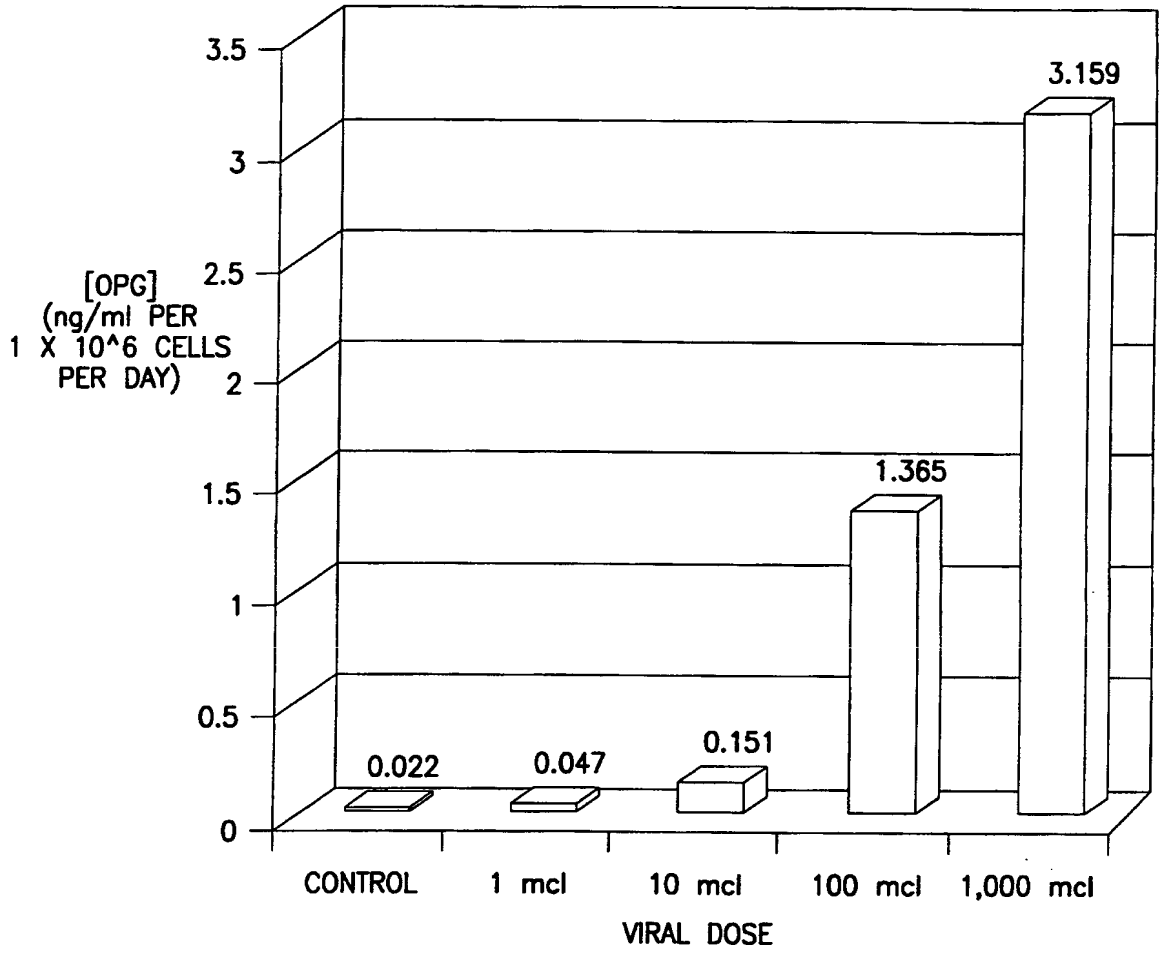
3.5
3
2.5
2
1.5
1
0.5
0
[OPG]
(ng/ml PER
1 X 10^6 CELLS
PER DAY)
0.022
0.047
0.151
1.365
3.159
CONTROL
1 mcl
10 mcl
100 mcl
1,000 mcl
VIRAL DOSE

FIG. 4

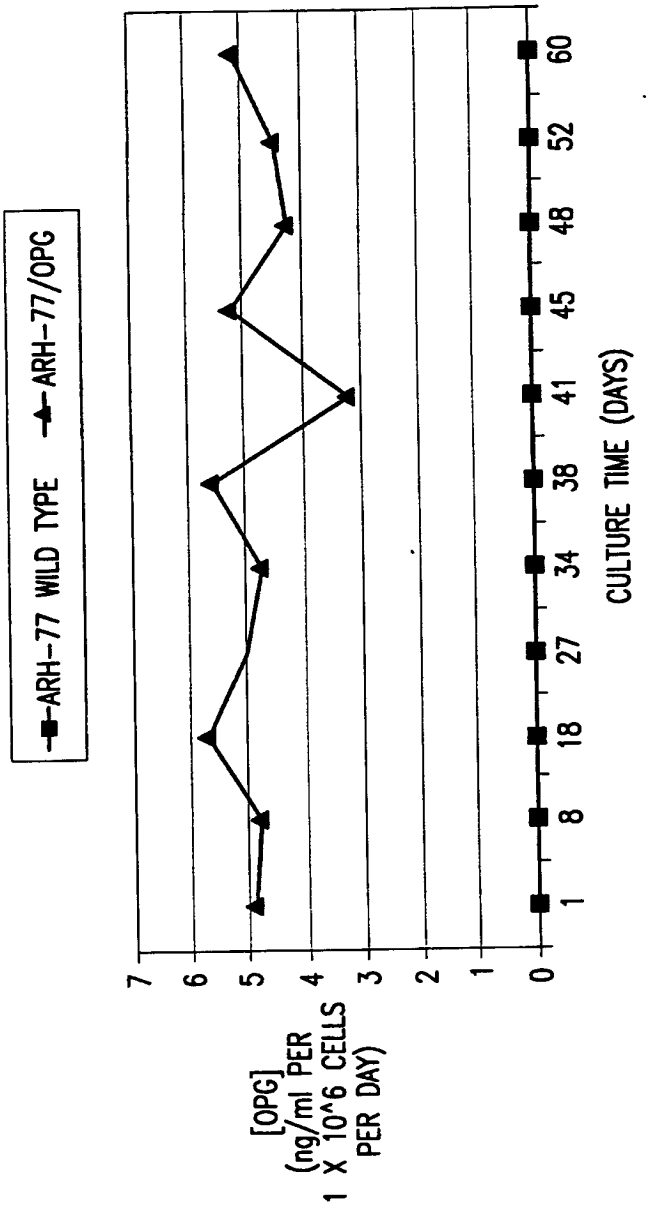
ARH-77 WILD TYPE
ARH-77/OPC
[OPC]
(ng/ml PER
1 X 10^6 CELLS
PER DAY)
0
1
2
3
4
5
6
7
1
8
18
27
34
38
41
45
48
52
60
CULTURE TIME (DAYS)

FIG. 5

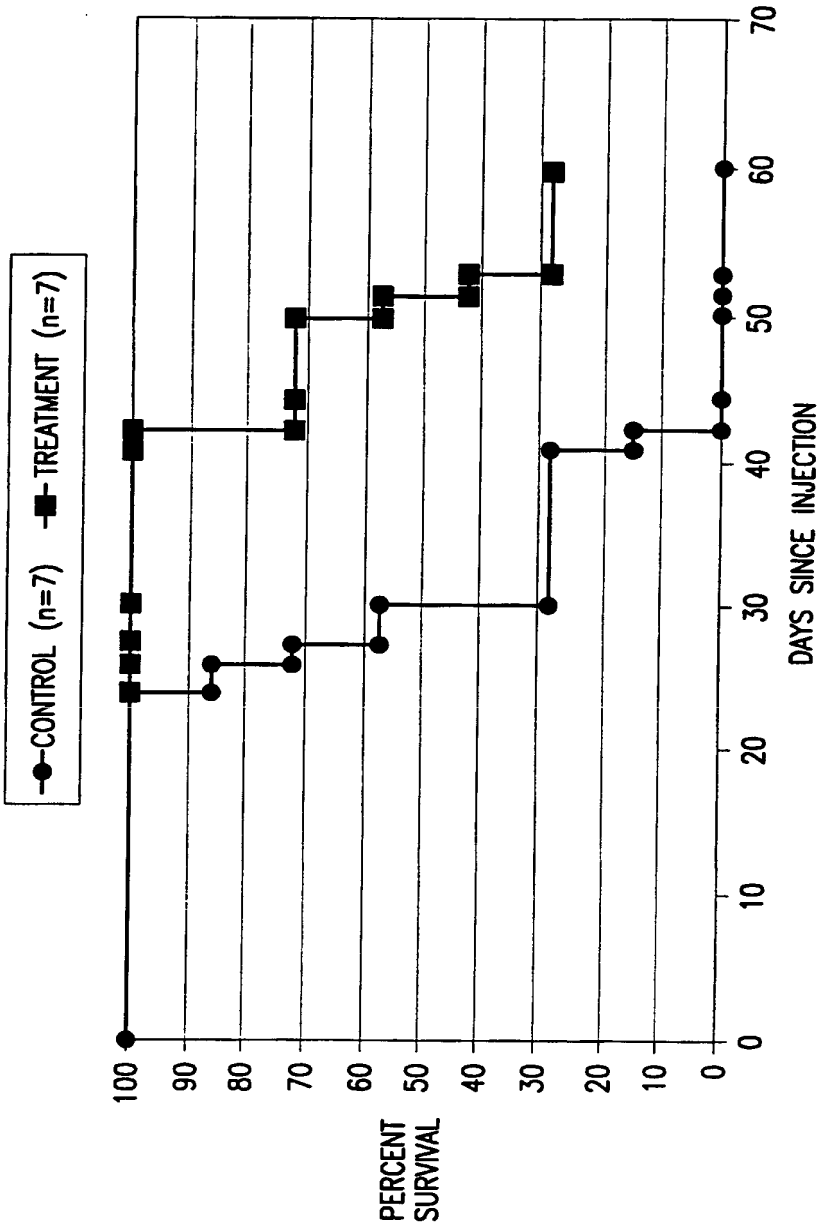
CONTROL (n=7)
TREATMENT (n=7)
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
DAYS SINCE INJECTION

FIG. 6

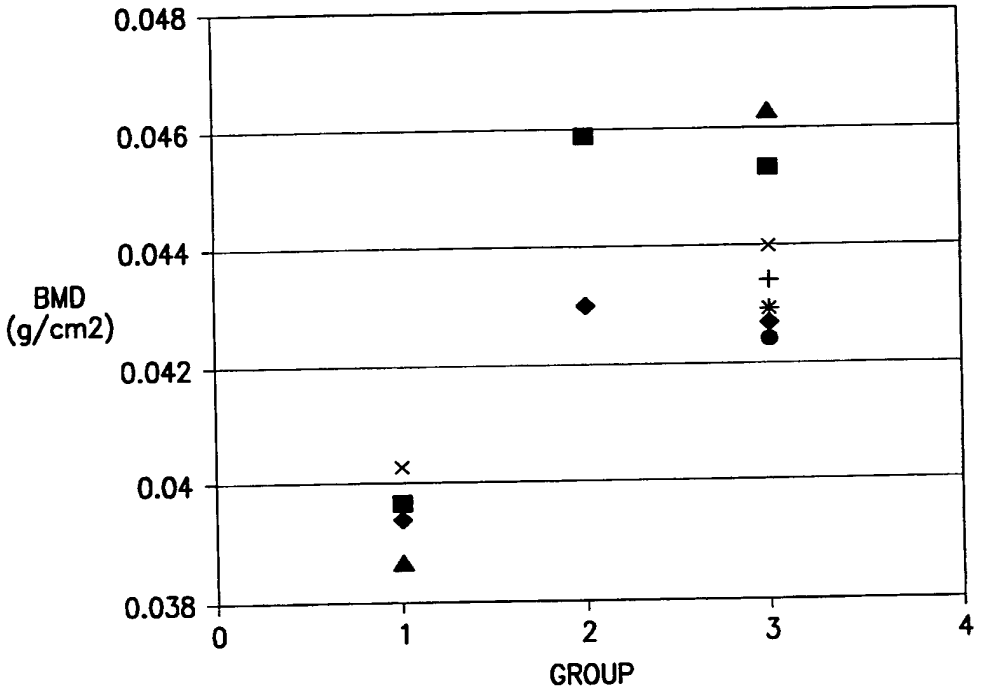

FIG. 7

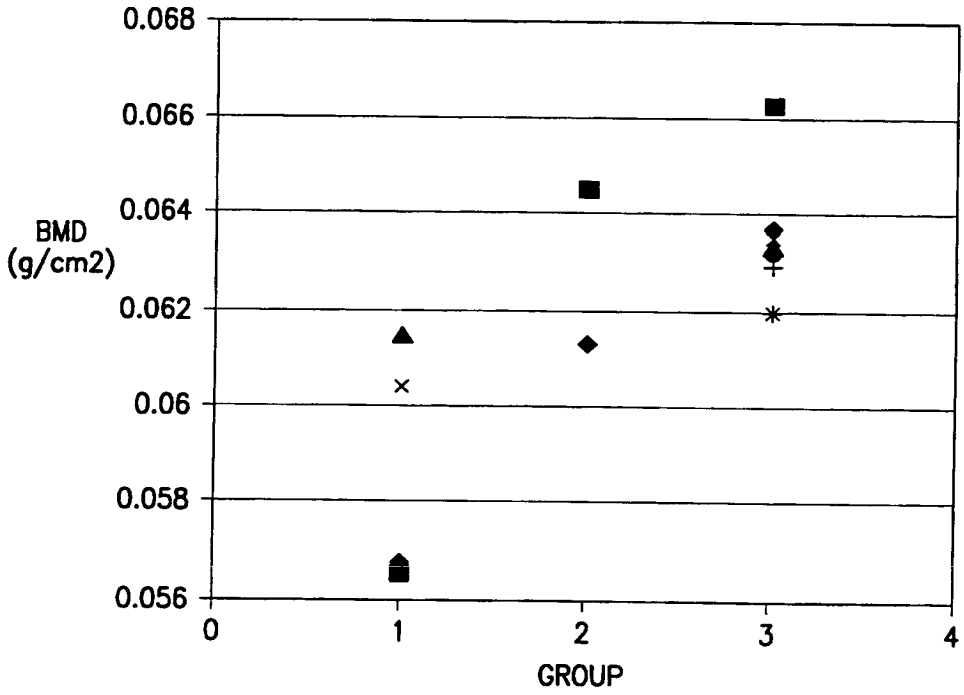

FIG. 8

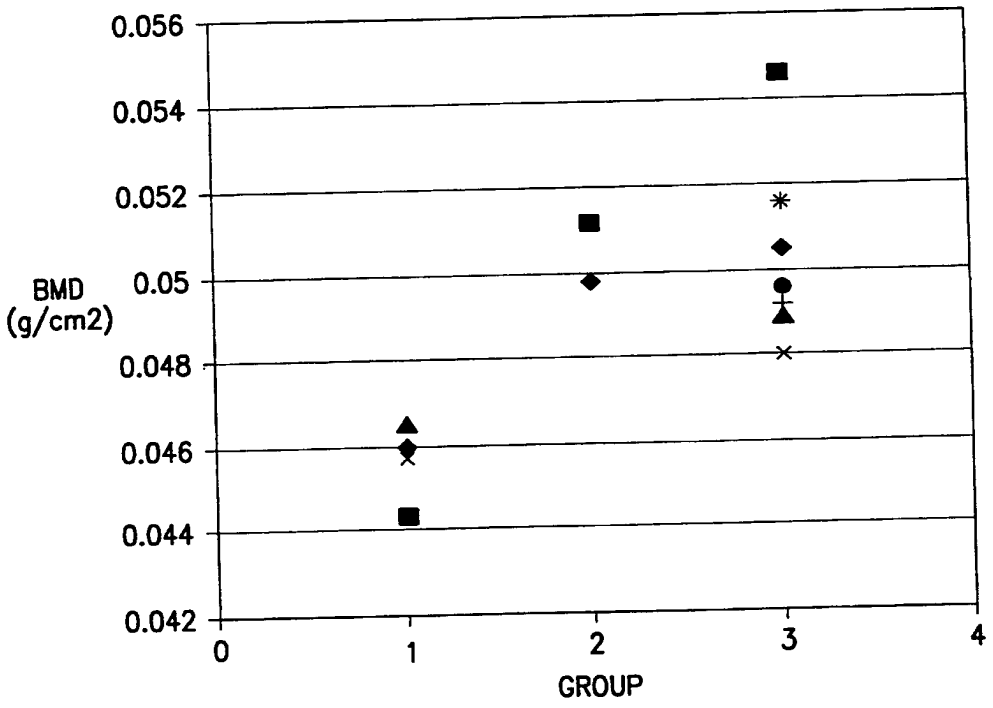

FIG. 9

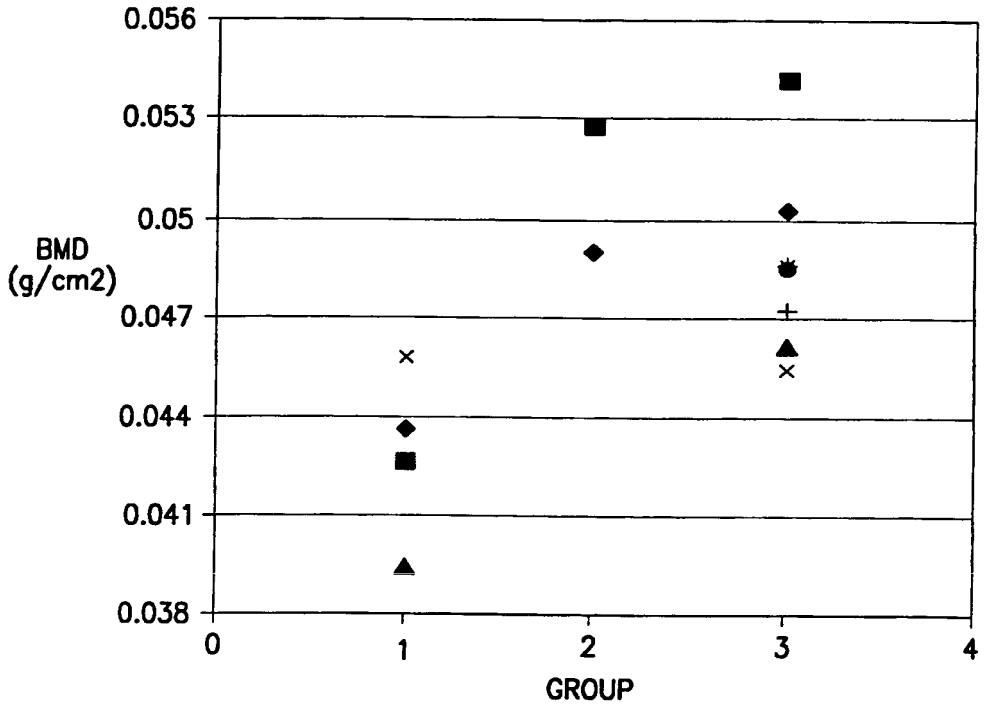

FIG. 10

SEQUENCE LISTING

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Doran, P.M.
Riggs, B.L.
Khosla, S.
Russell, S.T.

<120> Use of DNA encoding osteoprotegerin to prevent or inhibit metabolic bone disorders

<130> 150.197WO1

<150> 60/151,415
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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 00/23755

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/10 A61K48/00 A61K35/28 C12N15/861 C12N15/867
A61P19/00 A61P19/08 A61P19/10 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	EP 0 784 093 A (AMGEN INC) 16 July 1997 (1997-07-16) abstract page 2, line 50 -page 3, line 6	7,8, 21-23 1-6,9-20
A	SIMONET W ET AL: "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density" CELL,US,CELL PRESS, CAMBRIDGE, NA, no. 89, 18 April 1997 (1997-04-18), pages 309-319, XP002077048 ISSN: 0092-8674 cited in the application the whole document	1-23

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 January 2001	24/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Niemann, F

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 00/23755

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SMITH: "VIRAL VECTORS IN GENE THERAPY" ANNUAL REVIEW OF MICROBIOLOGY,US,ANNUAL REVIEWS INC., PALO ALTO, CA, vol. 49, 1995, pages 807-838, XP002091784 ISSN: 0066-4227 the whole document ---	7,8, 21-23
A	HESS P: "GENE THERAPY. A BRIEF REVIEW" CLINICS IN LABORATORY MEDICINE,GB,W.B. SAUNDERS CO., LONDON, vol. 16, no. 1, 1 March 1996 (1996-03-01), pages 197-211, XP000653352 ISSN: 0272-2712 the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 00/23755

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0784093 A	16-07-1997	AU 710587 B	23-09-1999
		AU 1468697 A	17-07-1997
		BG 101813 A	30-09-1998
		CA 2210467 A	03-07-1997
		CN 1182452 A	20-05-1998
		CZ 9702538 A	17-03-1999
		DE 19654610 A	26-06-1997
		EP 0870023 A	14-10-1998
		FR 2742767 A	27-06-1997
		GB 2312899 A,B	12-11-1997
		HU 9801122 A	28-08-1998
		JP 11503616 T	30-03-1999
		NO 973699 A	21-10-1997
		NZ 326579 A	28-01-1999
		PL 321938 A	05-01-1998
		SK 110797 A	12-07-1999
		TR 970550 A	21-07-1997
		WO 9723614 A	03-07-1997
		US 6015938 A	18-01-2000